IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor:	Ramesh, et al.	Docket No.:	3802-068-27 CIP (306229-65)
Serial No.:	10/743,813	Confirmation No.:	1728
Filing Date:	December 24, 2003	Art Unit:	1635
For:	METHODS AND REAGENTS FOR THE ENHANCEMENT OF VIRUS TRANSDUCTION IN THE BLADDER EPITHELIUM	Examiner:	Schnizer, R.A.

AFFIDAVIT UNDER 37 CFR 1.132

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Commissioner for Patents
P.O. Box 1450
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Dear Sir:

Ralph

I, DAVID FREY, being duly sworn, depose and state:

1. I understand that the Patent Application was filed on December 24, 2003, with the United States Patent & Trademark Office by Piper Rudnick LLP, and was accorded Serial No. 10/743,813.

2. Nagarajan Ramesh, Bahram Memarzadeh, DeChao Yu and I are co-inventors of the above-identified patent application, and the inventors of the subject matter described and claimed therein.

3. Throughout the time period from March 16, 1998 to present, I have held the titles of Associate Director and Director, Process Development, and have been supervising research and development projects related to product formulation and administration, as well as development of downstream manufacturing processes.

3. In my role Cell Genesys Inc., I supervised and worked on a project directed to methods of treating the luminal surface of the bladder to enhance infection with an oncolytic virus.

4. One aspect of the project was directed to a method for treating cancer of the bladder by either contacting the luminal surface of the bladder with a pretreatment composition comprising a transduction enhancing agent and subsequently contacting the luminal surface of the bladder with a composition comprising an oncolytic virus or contacting the luminal surface of the bladder with a composition comprising the combination of an oncolytic virus and a transduction enhancing agent.

5. A large number of detergents including standard agents such as polymer-based detergents (i.e. Tweens) and mono-, di-, or poly-saccharides having a lipophilic substituent were tested to evaluate their efficacy as transduction enhancing agents. The present invention is based on the results of these studies.

6. The invention relies on the use of a disaccharide, having a lipophilic substituent to enhance adenoviral transduction of the bladder epithelium. The invention is also directed to methods for transducing the bladder epithelium and for treating superficial cancer of the bladder derived from the bladder epithelium, based on pretreatment of the bladder with a transduction enhancing agent or by co administration of an oncolytic virus and a transduction enhancing agent.

7. A number of studies were performed under my supervision to evaluate the use of conditionally replication-competent oncolytic viruses for intravesicular therapy of bladder cancer. The results indicated that a class of compounds is effective for pretreatment of the bladder urothelium, permitting efficient adenoviral infection, by "permeabilizing" (not dissolving) a "mucous" membrane composed of the glycosaminoglycan (GAG) layer, which is not a cell membrane. The data disclosed in the subject patent application are later published (Ramesh et al., Mol. Ther. 10(4):697-705, 2004, **copy attached hereto as Exhibit A**).

8. Ramesh et al., demonstrate that intravesicular instillation of 0.1% DDM or SDS for 5 minutes into rat bladders, followed by infection with 10^{10} VP, resulted in greater than 90% transduction of the urothelial cell layer within 15 minutes after viral exposure. Bladders were

rinsed with PBS following treatment. (See, page 702 of Ramesh et al., Mol. Ther. 10(4):697-705, 2004.

9. Consistent with the experiments performed in our laboratory in support of the present invention, Ramesh et al. showed that when 10^{10} VP were coformualted with 0.1% DDM and retained in the bladder for 20 minutes, 70-80% transduction resulted and when the mixture was retained in the bladder for 45 minutes, 100% transduction resulted, when evaluated 48 hours after treatment.

10. The data is summarized in paragraph [0249] of the published patent application (US Patent Pub. No. 20040176318) which states the following: "formulating Ad-βgal virus with different concentrations of Dodecyl-β-D-Maltoside resulted in a linear Adenovirus transduction rate (0.1 % > 0.05 %> 0.025 %). For the 20 min. instillation, 0.1% DDM formulated with Ad-βgal virus resulted in about 80% gene expression in bladder, while 0.05% DDM resulted in about 40 % gene expression. But with 45 min. instillation, all animals with 0.05 % - 0.2 % DDM with Ad-βgal virus had 100 % gene expression in mice bladder. A 10 min. instillation for this formulation method, however, did not achieve an acceptable transduction rate. It was also found that gene transduction could be achieved with a 10 min. virus instillation after DDM pretreatment."

11. The results of this study illustrate that DDM can be formulated with Ad-βgal virus resulting in effective transduction in a mouse bladder model and that the time the bladder is exposed to a transduction enhancing agent such as DDM will affect the extent of transduction and that "with a DDM pretreatment, the time for virus instillation can be decreased to 10 min. from 45 min." (See, e.g., paragraph [0250] US Patent Pub. No. 20040176318).

12. A table comprising the transduction efficacy of a large number of compounds is attached hereto as **Exhibit B**. A ++++ transduction efficacy is described in Ramesh et al. as corresponding to greater than 75% of the luminal urothelium cells exhibiting beta galactosidase activity based on staining. A +++ transduction efficacy is described in Ramesh et al. as corresponding to 50 - 75% of the luminal urothelium cells exhibiting beta galactosidase activity

based on staining. A ++ transduction efficacy is described in Ramesh et al. as corresponding to 25 - 50% of the luminal urothelium cells exhibiting beta galactosidase activity based on staining. A + transduction efficacy is described in Ramesh et al. as corresponding to 10 - 25% of the luminal urothelium cells exhibiting beta galactosidase activity based on staining and a 0 transduction efficacy is described in Ramesh et al. as corresponding to 0 - 10% of the luminal urothelium cells exhibiting beta galactosidase activity based on staining.

13. Consistent with the data in the instant application as filed, all of the test compounds which facilitated a ++++ transduction efficacy are disaccharides or sulfates with an n-dodecyl (C12) side chain, with the exception of 6-cyclohexylhexyl-beta-D-maltoside.

14. All of the test compounds which facilitated a +++ or ++ transduction efficacy are disaccharides with a n-tridecyl (C13) or n-tetradecyl (C14) side chain or a polyether with an n-dodecyl (C12) side chain.

15. All of the test compounds which facilitated a + transduction efficacy are disaccharides or sulfates with a have an n-decyl (C10) side chain.

16. All of the compounds which facilitated a 0 transduction efficacy are monosaccharides with an n-dodecyl (C12) side chain, an n-octyl (C8) side chain, or a phenyl (C6) side chain; disaccharides with an n-octyl (C8) side chain, sulfates with an n-octyl (C8) or n-tetradecyl (C14) side chain; glycerol-based agents, Tween 20, Tween 80 or PBS.

17. A graphic depiction of the disaccharide and sulfate data is attached hereto as **Exhibit C.**

18. I reviewed Conner et al. (Gene Therapy 8:41-48, 2001), which indicates that the authors tested the effect of various detergents on viral transgene expression using a replication-deficient adenovirus containing the beta gal gene *in vivo* in rats. The rats received intravesicular administration of rAd-beta gal in a formulation containing 3.5×10^{10} viral particles and various detergents. Transgene expression was evaluated at 48 hours by a determination of LacZ expression in the entire luminal surface of the bladder. The viral particle/detergent composition

was left in the bladder for 45 minutes. Levels of viral gene expression were characterized on a scale of 0 to (++++), based on the percentage of the urothelium exhibiting beta gal. expression at 48 hours post virus administration.

19. It is my understanding that the Patent Office relies on Conner et al. (Table 1; page 42) as teaching that adenoviral infection of the urothelium could be improved by treatment of the urothelium with octyl-beta-D-glucopyranoside together with replication-deficient adenovirus. Conner et al. indicates the level of observed viral transgene expression upon infection with replication-deficient adenovirus diluted in octyl-beta-D-glucopyranoside was (++), indicated to correlate with 50-75% transgene expression over the luminal surface of the bladder. The concentration of octyl-beta-D-glucopyranoside is not disclosed in Conner et al., and the table indicates that viral stability was not determined.

20. If I relied on what I learned from reading Conner et al., I would have tested monosaccharides with C8 side chains, e.g., octyl-beta-D-glucopyranoside, shown in Table 1 of Conner et al., in order to enhance transduction of the bladder epithelium.

21. Contrary to the results presented in Conner et al., when alkyl monosaccharides or compounds with C8 side chains were tested in our laboratory as candidate transduction enhancing agents, no transduction was observed.

22. In contrast, when disaccharide and sulfate transduction enhancing agents with a side chain of 12 carbons were tested in our laboratory, transduction was observed at the ++++ level.

23. Conner et al. do not describe pretreatment of the bladder prior to exposure to virus or the concentration of detergent in the rAd-beta gal/detergent formulations described in the publication. Similarly, Conner et al., does not describe replication competent virus.

24. The current invention is based on the utility and efficacy of a group of bladder transduction enhancing agents and the enhanced efficacy of transduction with an oncolytic virus when the bladder is pretreated with the enhancing agent.

25. Prior to the present invention the efficacy of disaccharide and sulfate transduction enhancing agents with a side chain of 12 or more carbons was not known in the art.

26. The instant patent application describes a method for transduction of cells of the bladder epithelium for use in treating superficial bladder cancer derived from the bladder epithelium, exemplified by pretreatment of the luminal surface of the bladder with a transduction enhancing agent (such as described above) and a replication competent oncolytic virus comprising a urothelium-specific promoter such that cells of the bladder epithelium are transduced. By way of example a specific recombinant adenovirus, CG8840, is described. The invention is not limited to CG8840 and replication competent oncolytic viruses with different promoters are also effective in transduction of cells of the bladder epithelium.

27. Ar20-1004 is a replication competent adenovirus modified to express the murine cytokine granulocyte macrophage colony stimulatory factor (GM-CSF). In Ar20-1004 the adenoviral E1a promoter of wild type adenovirus is replaced with the human E2F-1 promoter. When rat bladders were pretreated with a transduction enhancing agent, dodecyl maltoside (DDM), prior to transduction with Ar20-1004, the level of GM-CSF detected in rat urine was over 18 times the amount detected when bladders were not pretreated prior to transduction. **See Exhibit D.**

28. Ar20-1004 has been shown to effectively transduce cells of the bladder epithelium. The extent of transduction of the bladder epithelium by Ar20-1004 is significantly enhanced when the luminal surface of the bladder is pretreated with a transduction enhancing agent, exemplified by dodecyl maltoside, a disaccharide with a C12 side chain.

29. Given the success of preclinical studies with Ar20-1004 and CG0070 (as summarized in Ramesh et al., Clin. Cancer Res. 2006, 12(1) 305 (copy provided herewith as **Exhibit E**), a phase I human clinical study was initiated.
In the clinical protocol, the bladders of all patients were pretreated with dodecyl maltoside, prior to transduction with CG0070, a virus which only differs from Ar20-1004 in that it produces human instead of rat GM-CSF. Patients were assessed for adverse events and laboratory

measures of toxicity. Cystoscopic examination of 3 patients day 8 after treatment with 10^{12}vp revealed inflammation and tumor regression. A complete response at week 12+ after treatment was reported in a patient with Ta TCC treated with 10^{12}vp. The results provide support for efficacy of the claimed transduction enhancing agents in transduction of the bladder epithelium with two oncolytic adenoviruses which differ in structure and mode of infectivity

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signed at _____, _____, this 8th day of February, 2007.

Ralph DAVID FREY

STATE OF CA

COUNTY OF San Mateo

Before me, a Notary Public for said County, personally appeared Ralph DAVID FREY, known to me to be the person who executed the foregoing affidavit and acknowledged it to be his act and deed.

Witness my hand and seal this 8th day of February, 2007

AILEEN ANGELES DAYAO
Commission # 1656506
Notary Public - California
San Mateo County
My Comm. Expires Apr 7, 2010

doi:10.1016/j.ymthe.2004.07.002

ARTICLE

Identification of Pretreatment Agents to Enhance Adenovirus Infection of Bladder Epithelium

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Available online 13 August 2004

Adenovirus has been used widely as a gene transfer vector in the laboratory and clinic for the purpose of gene therapy. Conditionally replication-competent oncolytic adenoviruses are capable of multiplying up to a thousand fold in target cells, a property that might prove to be of tremendous potential in the area of cancer therapy. Intravesicular therapy of refractory superficial bladder cancer employing an oncolytic adenovirus would allow for local administration and efficient delivery of virus to bladder tumor. The glycosaminoglycan layer on the surface of the bladder urothelium acts as a nonspecific antiadherence barrier and may be a significant roadblock to efficient infection of the urothelium by adenoviruses. Several laboratories have investigated the potential utility of bladder pretreatment with chemical agents to enhance the adenovirus infection of bladder urothelium but with limited success. A class of compounds has been identified that is effective for pretreatment of urothelium, permitting efficient adenoviral infection. In a murine model, pretreatment of the bladder with 0.1% dodecyl-β-D-maltoside (DDM) or sodium dodecyl sulfate (SDS) for 5 min resulted in >90% transduction of the urothelial layer within 15 min after exposure to a replication-defective adenovirus compared to ≤5% transduction in untreated bladders. DDM could be coformulated with adenovirus, and complete transduction of the urothelium was achieved following retention of the admixture in the bladder for 45 min. A similar enhancement of adenoviral infection following pretreatment of bladder with DDM and SDS was observed in a rat model. The use of these compounds may facilitate the development of adenovirus-based therapy for bladder cancer.

Key Words: bladder; pretreatment; adenovirus; glycosaminoglycan; tight junction

INTRODUCTION

Bladder cancer is the fourth most common malignancy in males in the United States and Europe, and local recurrence after standard therapy is common [1]. Despite aggressive local therapy, approximately 25–30% of patients who present with superficial bladder tumors will progress into muscle-invasive disease [2–4]. It is essential that novel therapies be developed that could interfere with the progression of superficial tumors into the more invasive phenotype and, if possible, completely eradicate the tumors.

Adenoviral vectors have been extensively used as gene transfer vectors for gene therapy in both experimental and clinical settings [5–9]. The use of conditionally replication-competent oncolytic viruses in the treatment of a variety of cancers is being explored [10–12]. A uroplakin II-positive bladder tumor-specific oncolytic adenovirus (CG8840) has been developed for the treatment of transitional cell carcinoma [13]. Intravesical instillation permits local administration and efficient delivery of the oncolytic virus to the tumor with minimal systemic exposure. For successful treatment of bladder cancer, efficient infection of a large percentage of the urothelial surface must be achieved so that subsequent replication and spread of the virus could eradicate tumor tissue. The apical membrane of the urothelium along with the urothelial plaques and tight junctions is believed to serve as one of the tightest and most impermeable barriers in the body [14–17]. Further, the polyanionic glycosaminoglycan (GAG) layer overlying the urothelium acts as an additional barrier, thus preventing adherence of infectious agents to the luminal urothelium [18–21]. Use of reagents that would remove or attenuate the function of the GAG layer would allow access of adenovirus to the

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EXHIBIT A

ARTICLE doi:10.1016/j.ymthe.2004.07.002

TABLE 1: Enhancement of adenoviral transduction

Pretreatment agent	Transduction efficacy
Alkyl disaccharides[a]	
n-Dodecyl-β-D-maltoside (C12)[a]	++++
n-Dodecyl-α-D-maltoside (C12)	++++
Sucrose monolaurate (C12)	++++
6-Cyclohexylhexyl-β-D-maltoside (C12)	++++
n-Tridecyl-β-D-maltoside (C13)	+++
n-Tetradecyl-β-D-maltoside (C14)	++
n-Decyl-β-D-maltoside (C10)	+
n-Octyl-β-D-maltoside (C8)	0
Alkyl sulfates (ionic alkyl)[a]	
Sodium dodecyl sulfate (C12)	++++
Sodium decyl sulfate (C10)	+
Sodium octyl sulfate (C8)	0
Sodium tetradecyl sulfate (C14)	0
Sodium dodecyl benzene sulfonic acid (C12)	++
Alkyl (ether) alcohol	
Polidocanol	++
Polymeric surfactants	
Triton X-100	+
Poloxamers F68, F127	0
Tween 20, Tween 80	0
Alcohols	
0.1–3% Benzyl alcohol	0
10–30% Ethanol	+
Mixed agents	
DOTAP + cholesterol (*in vivo* gene shuttle)	0
0.1–0.2% Chlorpactin WCS-90 (oxychlorosene)	+++

Various agents were tested for their ability to enhance the infectivity of the bladder epithelium by a replication-incompetent adenovirus (Adβ-gal) following a short pretreatment with the agent. Forty-eight hours after virus infection, the fixed bladders were stained for the expression of β-galactosidase. Transduction efficiency was estimated based upon the percentage of the luminal urothelium exhibiting β-galactosidase activity. Transduction efficiency: (0) 0–10%, (+) 10–25%, (++) 25–50%, (+++) 50–75%, (++++) >75%. DOTAP, (*N*-[1-(2,3-dioleoyloxy)]-*N*,*N*,*N*-trimethylammonium propane methyl sulfate.

[a] The number of carbon chains in the compound is indicated in parentheses.

urothelial cell surface. Pretreatment of bladder with compounds such as ethanol, acetone, and HCl have led to some enhancement in infection of the urothelium by pox and adenovirus when used only at high concentrations that often result in significant irritation to the urothelium [19,20,22,23].

In the current study, we have investigated the use of several chemical compounds with specific structural features to overcome the permeability barrier imposed by GAG, which would permit efficient access for the virus to the urothelium, including the tumor cells.

FIG. 1. Enhancement of urothelial transduction by oxychlorosene pretreatment in the bladders of female mice. (A–D) Bladders were pretreated with 0.1% oxychlorosene or PBS and virus was instilled as described under Materials and Methods. Depicted are representative whole bladders from a single animal tested in each group ($n = 6$ per group). (E–H) Bladders were harvested, fixed, and stained with X-gal as described under Materials and Methods. Microscopic examination of the bladder showed that β-galactosidase expression was confined to the urothelium. (Original magnification: 20×.)

FIG. 2. Enhancement of urothelial transduction by SDS pretreatment in the bladders of female mice. (A–C) Bladders were pretreated with 0.1% SDS and the indicated concentration of the virus as described under Materials and Methods. Depicted are representative whole bladders from a single animal tested in each group ($n = 6$ per group). Bladders were harvested, fixed, and stained with X-gal as described under Materials and Methods. (D–F) Histological examination of the bladder showed that β-galactosidase expression was confined to the urothelium. (Original magnification: 20×.)

FIG. 3. Enhancement of urothelial transduction by DDM pretreatment in the bladders of female mice. (A–C, H) Bladders were pretreated with 0.1% DDM and the indicated concentration of virus as described under Materials and Methods (pretreatment agent/virus volume: A–C, 100 μl; H, 50 μl). Depicted are representative whole bladders from a single animal tested in each group ($n = 6$ per group). Bladders were harvested, fixed, and stained with X-gal as described under Materials and Methods. (D–G) Histological examination of the bladder showed that β-galactosidase expression was confined to the umbrella cells of urothelium with no expression observed in lamina propria. (Original magnification: D–F, 20×; G, 40×.)

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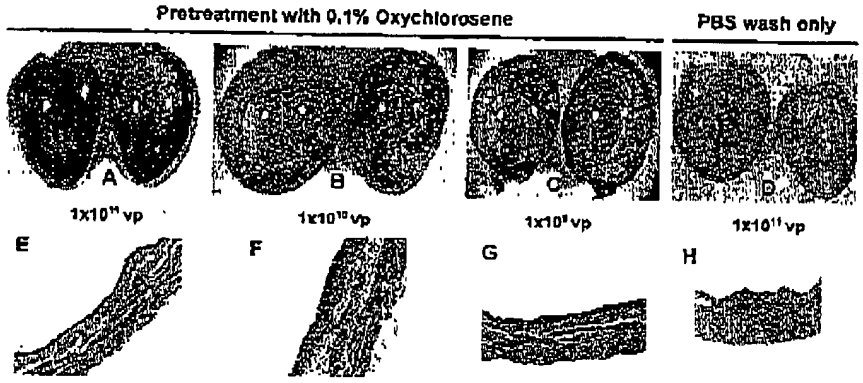

FIG. 1.

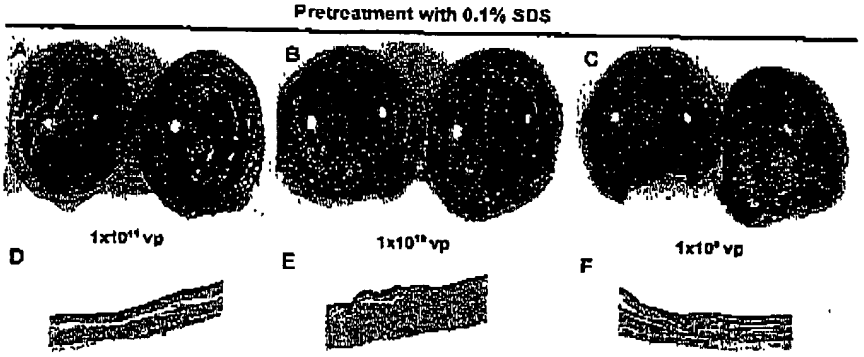

FIG. 2.

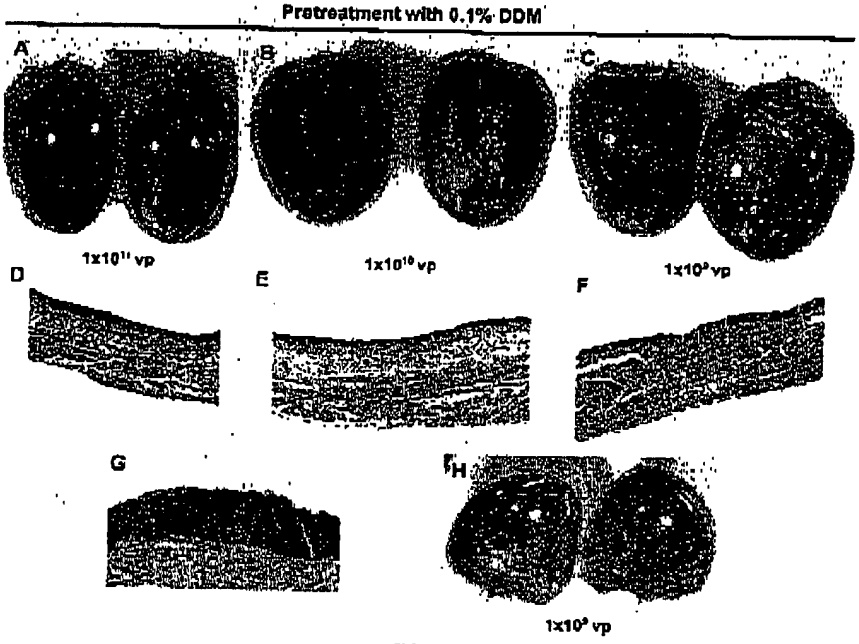

FIG. 3.

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doi:10.1016/j.ymthe.2004.07.002

RESULTS

We investigated several ionic and nonionic organic surfactants for their potential to remove or attenuate the barrier function of the GAG layer (Table 1). Pretreatment of the bladder with several of the compounds for a short duration (5 min) permitted complete transduction of the superficial layer of the urothelium by adenovirus, whereas others were much less effective. Details of the most effective compounds for enhancing adenovirus infection of bladder urothelium are presented below.

Sodium Oxychlorosene

A 5-min pretreatment of the bladder with 0.1 to 0.2% oxychlorosene followed by infection with adenovirus (10^{11} vp) resulted in complete transduction of the bladder epithelium (Fig. 1). The degree of transduction of the urothelium was significantly lower when viral concentration was decreased to 10^9 vp (Fig. 1), and we saw lot-to-lot inconsistency in the degree of transduction. Microscopic evaluation of the urothelium in bladder sections pretreated with 0.1% oxychlorosene revealed normal morphology. Longer pretreatment or treatment with higher concentrations of oxychlorosene resulted in significant submucosal edema, infiltration of neutrophils with mucosal erosion, and/or ulceration (data not shown), indicating significant potential for irritation. In addition, *in vitro* compatibility studies showed that adenovirus was incompatible with oxychlorosene, suggesting that residual oxychlorosene remaining in the bladder after pretreatment could potentially decrease the transduction of the urothelium (Table 2). Extensive washes with PBS after pretreatment or the use of a reducing agent wash, such as sodium metabisulfite, did not improve the consistency of transduction (data not shown).

Sodium Alkyl Sulfate Compounds

Pretreatment of the bladder with several of the alkyl sulfate compounds resulted in a significant enhancement in the degree of adenovirus infection of the urothelium (Table 1). For example, a 5-min pretreatment of the bladder with 0.1% SDS resulted in complete transduction of the bladder urothelium using 10^{11} vp of adenovirus and a 15-min incubation (Fig. 2), with no visible histopathological changes suggestive of irritation. Complete transduction of the urothelial layer was also achieved at adenoviral doses as low as 10^9 vp retained in the bladder for 15 min. However, *in vitro* compatibility studies showed that virus integrity was significantly affected when coformulated with ≥0.02% SDS and held for 1 h at 25°C (data not shown). The virus lost its infectivity as assessed by plaque assay (Table 2). These findings suggest that SDS is not an optimal pretreatment agent since it was found to be difficult to wash SDS completely from the bladder prior to the instillation of the adenovirus. Use of the alkyl sulfates containing C8, C10, or C14 chains did not result in significant enhancement of viral infectivity above the control levels compared to the C12-derived SDS (Table 1).

Alkyl Disaccharide Compounds

Enhancement of the infectivity of the urothelium by adenovirus was also achieved with a 5-min pretreatment of the bladder with 0.1% *n*-dodecyl-β-D-maltoside (DDM), which resulted in complete transduction of the bladder epithelium with a 10^{11} vp dose of adenovirus (Fig. 3). DDM also enhanced urothelial infection at a lower dose of 10^9 vp using a bladder retention time of 15 min (Figs. 3A–3C). In these studies 100 μl of the pretreatment/virus solution was instilled intravesically in the bladder. To address the issue of the effects of physical parameters such as pressure and volume on the transduction efficiency, we performed additional studies with lowered reagent volume. The enhancement in adenovirus transduction efficiency was similar when the reagent volume was reduced to 50 μl (Fig. 3H). *In vitro* compatibility studies with 0.1% DDM showed that DDM did not affect the infectivity potential of the adenovirus (Table 2). *In vivo* studies showed that the coformulated virus could transduce the bladder effi-

TABLE 2: *In vitro* compatibility of adenovirus with pretreatment agents

Pretreatment agent	Temperature	vp/pfu
0.1% Chlorpactin WCS-90 (oxychlorosene)	25°C	974
0.1% Chlorpactin WCS-90 (oxychlorosene)	37°C	24,286
0.1% Polidocanol	25°C	20
0.1% Polidocanol	37°C	17
0.1% *n*-Dodecyl-β-D-maltoside	25°C	16
0.1% *n*-Dodecyl-β-D-maltoside	37°C	15
0.1% Sodium dodecyl sulfate	25°C	np
0.1% Sodium dodecyl sulfate	37°C	np

Plaque assay was performed after incubating 2.5×10^{11} vp of Adβ-gal virus (initial vp/pfu: 22) at the indicated temperature and concentration of the pretreatment agent for 1 h. Higher vp/pfu ratios indicate inactivation of virus. np, no plaques detected.

doi:10.1016/j.ymthe.2004.07.002

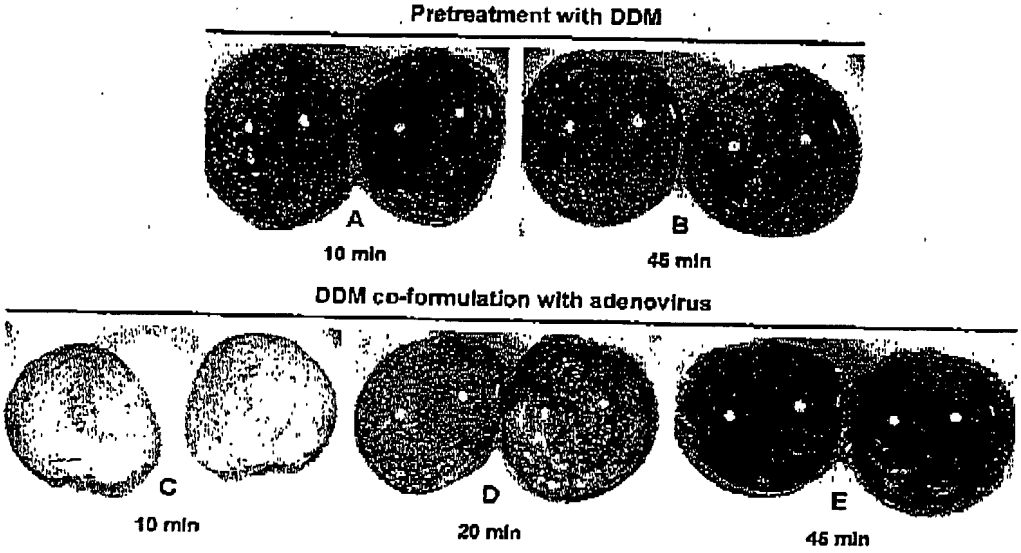

FIG. 4. Comparison between DDM pretreatment and DDM co-formulation for enhancement of adenovirus infection of mouse bladder urothelium. (A, B) X-gal staining of bladder after pretreatment of the bladder with 0.1% DDM for 5 min followed by adenovirus infection (10^{10} vp) for either 10 or 45 min. (C–E) X-gal staining of the bladder after treatment with adenovirus coformulated with DDM. Adenovirus (10^{10} vp) was mixed with DDM (0.1% final concentration) prior to the instillation into the bladder lumen. The mixture was retained in the bladder for 10, 20, or 45 min.

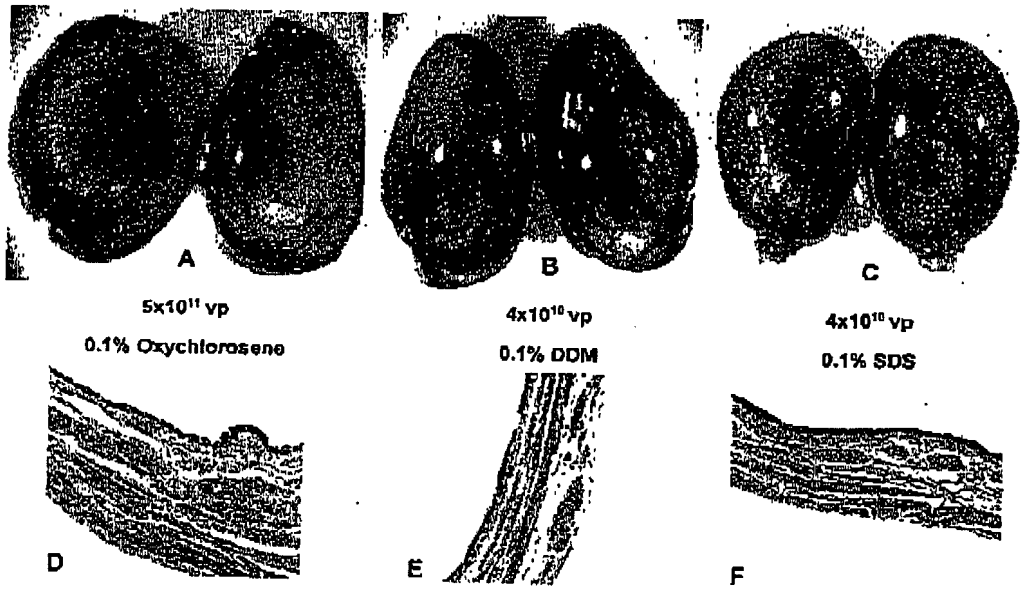

FIG. 5. Enhancement of urothelial transduction in the bladders of female rats. (A–C) Bladders were pretreated with the indicated agent followed by virus instillation as described under Materials and Methods. Animals were euthanized 48 h after treatment and bladders were harvested, fixed, and stained with X-gal as described under Materials and Methods. Depicted are representative whole bladders from one animal in each group (3 animals tested per group). (D–F) Histological examination of the bladder showed that β-galactosidase expression was confined to the urothelium. (Original magnification: 20×.)

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doi:10.1016/j.ymthe.2004.07.002

ciently albeit after a longer retention time. Intravesical instillation of the adenovirus (10^{10} vp) coformulated with 0.1% DDM, when retained in the bladder for 20 min, resulted in 70–80% transduction of urothelium and 100% transduction with retention for 45 min (Fig. 4). Histopathological examination of the bladder showed that DDM was well tolerated when used alone or when coformulated with adenovirus. Microscopic examination of the β-galactosidase-stained sections of bladder at higher magnifications showed that the adenovirus transduction was restricted to the urothelial layer and the cells of the inner layers were not transduced (Figs. 3D–3G).

In studies in which DDM (C12) was compared with other alkyl chain compounds (C8–C14), maximum enhancement of adenoviral transduction was observed with the C12 compound (DDM) (Table 1). Pretreatment of bladder with other dodecyl disaccharides such as cyclohexylhexyl-β-D-maltoside or sucrose laurate under similar conditions also resulted in enhanced transduction of urothelium comparable to pretreatment with 0.1% DDM (Table 1). Pretreatment with both the α- and the β-isomer of DDM resulted in similar levels of enhancement in the transduction of urothelium. Other compounds, such as phenyl glucoside and glycerol laurate, did not demonstrate significant activity (data not shown).

Pretreatment studies were also performed in rats with oxychlorosene, DDM, and SDS, and the transduction levels of the urothelium with adenovirus were determined (Fig. 5). Pretreatment with 0.1% oxychlorosene resulted in >90% transduction of the urothelium in many bladders, but we observed significant inconsistency in the transduction frequency of the urothelium, presumably due to residual oxychlorosene in the bladder. In contrast, complete and consistent transduction of the urothelium was achieved after pretreatment of the bladder with 0.1% DDM or SDS for only 5 min.

Evaluation of Irritative Potential of Potential Pretreatment Agents

Microscopic examination of the bladders treated with either 0.1% DDM or SDS revealed that pretreatment with these agents did not significantly affect the integrity of the urothelial layer. We observed occasional cases of mild ulceration, neutrophil infiltration, or edema but they appeared to be a result of the physical invasiveness of the catheterization procedure rather than due to the pretreatment agent. We observed no pretreatment-related deaths of the animals with either SDS or DDM.

Electron Microscopy Studies

We examined the urothelial surfaces of the bladders by electron microscopy (EM) to evaluate the ultrastructural changes in DDM-treated or saline-treated bladders. A densely stained contiguous GAG layer of uniform thickness was evident over the urothelial cells in saline-pretreated bladders (Fig. 6A). This layer was separated from the outer leaflet of the plasma membrane by an electron-lucent layer. Tight junctions, characteristic of epithelial cell layers, were well maintained between contiguous urothelial cells, preventing ruthenium red penetration between the cells (Fig. 6C). In contrast, a distinct GAG layer was absent on the urothelium of DDM-pretreated bladders and had been replaced by a spongy, noncontiguous and weakly stained GAG layer (Fig. 6B). In addition, the electron-lucent layer was not apparent in many parts of the urothelial surface. Disruption of tight junctions was also seen along the surface in the DDM-pretreated bladder as visualized by the penetration of the ruthenium red stain (Fig. 6D).

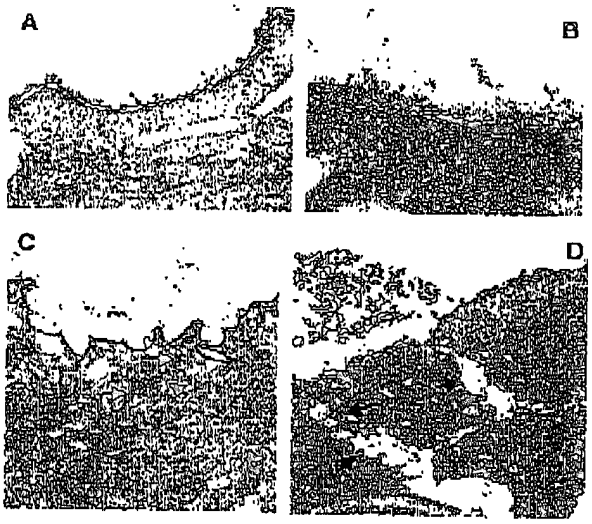

FIG. 6. Electron microscopy of urothelial layer of mouse bladder after DDM pretreatment. GAG on the urothelial surface following pretreatment with (A) saline or (B) 0.1% DDM. (C) The intact tight junction in saline-treated bladder and (D) the disrupted tight junction in 0.1% DDM-pretreated bladder are indicated by solid arrows.

DISCUSSION

The urothelial plaques are thought to perform several functions, including a strong role in the establishment of a permeability barrier for both water and solutes. Overlying this barrier is the layer of GAG on the luminal surface of the bladder that must be circumvented first to permit access to the urothelial surface for efficient transduction by viral vectors. The polyanionic molecules of the GAG layer form a polar shield with the highly ordered water molecules bound to its surface, thus blocking access to the urothelium [25]. The abrogation of the GAG layer by organic agents such as ethanol and acetone is thought

doi:10.1016/j.ymthe.2004.07.002

to occur via their mucin-degrading action. Protamine sulfate, a mucin-degrading agent, has been shown to disrupt the GAG layer through the formation of tight ionic bonds between its quaternary amine groups and the sulfated GAG, which then displaces the ordered water molecules [26]. Intravesical treatment with protamine sulfate and urea has been shown to lead to bladder epithelial desquamation, leading to the destruction of transitional cells [27]. Earlier studies have documented the use of several chemicals, including acids, organic agents, and detergents, to attenuate or remove the GAG layer to improve access to the urothelial surface [19,20]. Many agents were effective in breaking down the barrier, but often were irritating to the bladder at the effective concentrations. In the current study, several compounds have been identified that enhance the infection of the epithelial layer of the bladder by adenovirus and that are also well tolerated and could thus be potentially useful as pretreatment agents in humans.

Surfactants are broadly classified by their molecular and structural chemistry, charge, or behavior in solution. The block polymers, such as the polyoxyethylene, polyoxypropylene copolymer (poloxamer) polyethylene monolaurate (Tween), contain alternating hydrophilic–hydrophobic regions, which result in an almost even distribution of partial charges and in nonpolar interactions with solutes. The polar surfactants, such as DDM and SDS, consist of a hydrophilic head and a hydrophobic tail. The two portions are typically linked by a flexible ether bond that limits steric pressure during physical interaction. There are several variations of this class of surfactant that could affect the behavior of each chemical. The hydrophobic section of the molecule, generally an alkyl chain, can exist in a variety of lengths and can be either linear or branched.

The chemicals tested in this study for enhancing the infectivity of adenovirus *in vivo* in bladder epithelial cells can be classified into ineffective, medium, and strongly effective agents. Block copolymers, including polymeric surfactants with alternating hydrophilic–hydrophobic regions with short alkyl chains such as Triton X-100, were ineffective. The alkyl (ether) alcohol-containing block copolymers showed intermediate effectiveness in enhancing the transduction of the urothelium. The alkyl disaccharides with well-separated hydrophilic and hydrophobic regions, such as DDM, were the most effective agents. Within this class of compounds, the carbon chain length of the hydrophobic group seems to have an important effect on the enhancement of adenoviral transduction. Compounds containing a C12 alkyl chain (dodecyl) provided optimal effectiveness compared to those with a higher or lower number of carbon units. The effectiveness of the C12 alkyl chain (dodecyl) appears common between both disaccharide and sulfate detergent classes.

Ultrastructural examination of the urothelial surface revealed that complete removal of the GAG layer is apparently not essential for the efficient infection of the urothelium by adenovirus. As observed by EM, the GAG layer was not completely removed by 0.1% DDM pretreatment but had been replaced by a spongy noncontiguous layer, which may have provided access to the urothelial surface.

Adenoviruses are known to enter cells by receptor-mediated endocytosis that is initiated by the interaction of the knob of the fiber protein with a cell surface receptor called the coxsackievirus–adenovirus receptor (CAR) [28]. Structural and functional studies have shown that CAR forms homodimers between molecules located on adjacent cells and serves as a cell–cell adhesion molecule [29]. In polarized epithelial cells, the CAR is hidden beneath the tight junctions and not readily accessible for virus binding. Recent studies on the mechanism of adenovirus entry into polarized epithelium, such as airway epithelial cells, have demonstrated the requirement for the disruption of the tight junctions to gain access to the CAR [30,31]. Sequestration of CAR in tight junctions may thus limit virus infection across epithelial surfaces, including the urothelium, and the disruption of the tight junctions by the pretreatment agents could be an added component that permits efficient infection of the urothelium. DDM pretreatment resulted not only in the disruption of the GAG layer but also in the opening of the tight junctions. Thus, although the GAG layer was attenuated by DDM pretreatment, the opening of the tight junctions may have provided greater access of the CAR to adenovirus, resulting in enhanced transduction of the urothelium.

The mechanism of transduction enhancement by the tested agents in the current study has not been unequivocally established. Additional studies to determine the mechanism of action of DDM are currently in progress. It is also possible that these agents may enhance transduction via a non-CAR-mediated mechanism, as has been postulated for Syn3, a compound identified previously for the enhancement of adenovirus infection of bladder urothelium [21]. This seems unlikely, however, since the compounds identified in the present study enhance the transduction of the urothelium by adenovirus even when they are used only as pretreatment agents and not be present during the virus infection process.

Earlier studies with canarypox virus had shown significant differences in bladder epithelial transduction efficiencies following changes in the pressure and volume during intravesical instillation [23]. Our studies with lowered reagent/virus instillation volume (50 μl instead of 100 μl) did not result in lowered transduction efficiencies, indicating the absence of pressure- or volume-related effects on the infectability of bladder epithelium by adenovirus.

doi:10.1016/j.ymthe.2004.07.002

The ability to coformulate adenovirus with DDM provides for flexibility to either pretreat the bladder or, if preferred, instill the bladder simultaneously with a coformulation of virus and DDM. DDM may also facilitate the enhancement of transduction by other viral vector-mediated gene transfer to epithelial layers that are covered with GAG. A published toxicology study has shown that DDM is well tolerated in mice, rats, dogs, and monkeys when infused intravenously at and above the concentrations that were used intravesically in our studies [32].

In summary, in the present study, we have shown that pretreatment of the bladder with DDM, other dodecyl disaccharides, or SDS resulted in improved adenoviral infection of the bladder urothelium. These compounds are effective at low and apparently nonirritating concentrations. The short pretreatment times, coupled with minimal cellular damage to the urothelial cell layers, makes these agents attractive candidates for removal of potential barriers to adenoviral transduction in the bladder and possibly preferable to compounds currently employed in the field for a similar purpose.

Materials and Methods

Chemicals. Sodium oxychlorosene (Clorpactin WCS-90; Guardian Laboratories); DDM, sucrose monolaurate, cyclohexylhexyl-β-D-maltoside, decyl-β-D-maltoside, Triton X-100, Tween 20 and 80, polidocanol, SDS, and sodium dodecyl benzene sulfonic acid (Sigma Chemicals); *n*-dodecyl-α-D-maltoside (Incalco Pharmaceuticals); Gene shuttle (Qbiogene); dehydrated alcohol (Spectrum); benzyl alcohol (Aldrich); sodium tetradecyl sulfate (Omega); sodium decyl sulfate (Avacado Chemicals); poloxamers F68 and F127 (BASF); octyl-β-D-maltoside, tridecyl-β-D-maltoside, and tetradecyl-β-D-maltoside (Anatrace) were the reagents employed in the studies.

Adenoviral construct. A replication-incompetent adenovirus expressing β-galactosidase (Adβ-gal) was utilized as a marker virus in this study. It was derived from an E1A- and E1B-deleted human adenovirus type 5 virus and contained the lacZ reporter gene under the control of the cytomegalovirus early promoter. The adenovirus was expanded in 293 cells and purified by chromatography. Purified virus concentrations were determined in viral particles per milliliter and infectious particles by plaque assay as described earlier [24]. The concentration of the adenovirus preparation was 1×10^{12} vp/ml with a vp-to-pfu ratio of 22.

Pretreatment agent compatibility. For compatibility studies, 2.5×10^{11} vp of Adβ-gal virus (vp/pfu: 22) was mixed with the specified concentration of the pretreatment agent and incubated at the indicated temperature for 1 h after which the viability of the virus was determined by plaque assay as described earlier [24].

Gene transfer studies in mice and rats. Female Balb/c mice and Sprague–Dawley rats were used in these studies. All animals were housed according to institutional regulations for experimental animals. Balb/c mice (approximately 20 g) were anesthetized with isoflurane, and a 24-gauge catheter was introduced through the urethra into the bladder. The residual urine was emptied and the bladder was flushed three times with 100 μl each of PBS employing a 25-gauge needle inserted into the external end of the catheter. After the wash, bladder pretreatment was performed employing the various test reagents as follows: the pretreatment reagent was diluted to the desired concentration in PBS, and the bladder was quickly rinsed twice with 100 μl each of the reagent. After the rinsing steps, 100 μl of the pretreatment reagent was introduced via the catheter and retained in the bladder for 5 min, followed by an additional quick rinse with the pretreatment reagent. The bladder was then washed three times with 100 μl each of PBS. One hundred microliters of the β-gal-expressing adenovirus at the desired concentration was administered intravesically and retained in the bladder for the indicated time. Treatment was stopped by manual expression of the virus followed by a wash with PBS. Animals were permitted to recover from anesthesia, and bladders were harvested 48 h after treatment for evaluation of the effect of the pretreatment agent and the infection of the urothelium. Additionally, pretreatment and virus infection studies were also performed employing only 50 μl of the reagents instead of 100 μl. Other than the lowered reagent volumes all other procedures were similar to those of the 100 μl instillation studies.

A similar pretreatment and virus infection protocol was employed for studies with Sprague–Dawley rats with some modifications. Female Sprague–Dawley rats (approximately 200 g) were anesthetized with isoflurane and a 20-gauge catheter was introduced through the urethra into the bladder. The volume of reagents used at each of the washing, pretreatment, and virus infection steps was 400 μl.

β-Galactosidase expression analysis. Animals were euthanized 48 h after treatment, and bladders were filled with 100 μl of whole organ fixative *in situ* (2% neutral-buffered formalin, 2% glutaraldehyde, 2 mM $MgCl_2$, 10 mM PBS, pH 7.4). The bladder was immediately removed and immersed in whole organ fixative for 1 h. Thereafter, the bladder was incised longitudinally, rinsed in wash buffer (2 mM $MgCl_2$, 0.2% Triton X-100) overnight at 4°C, and stained with X-gal staining solution (4 mM potassium ferro/ferricyanide, 1 mM $MgCl_2$, 0.5 mg/ml X-gal in PBS). Following gross examination of the bladder, transduction efficiency was estimated based upon the average percentage of the superficial urothelium exhibiting X-gal staining in the histological sections at different levels and regions of the stained bladder. For histological examination of paraffin-embedded bladder, 5-μm-thick paraffin sections of the X-gal stained bladder were counterstained with hematoxylin.

Electron microscopy. Bladder tissues were fixed in a solution of 4% paraformaldehyde, 2.5% glutaraldehyde, and 0.1% ruthenium red in 0.1 M cacodylate buffer. Tissues were gradually dehydrated and cleared with propylene oxide. The tissues were then embedded in Epon/Araldite and both thick (glass knife) and ultrathin sections were cut (Diatome diamond knife). Sections were placed on grids and stained with uranyl acetate followed by lead citrate and examined on a Philips CM-EM. Images were captured on Kodak SO-163 film.

Acknowledgments

We thank A. J. Kaelne, T. Gire, J. Langer, Ho, and S. Ulufatuin the animal facility for outstanding technical assistance with animal surgery; G. Colbern for advice on animal studies; and S. Chattopadhyay for help with EM. We also thank P. K. Working, T. Harding, S. Police, L. C. Kellerman, L. Hawkins, and Y. H. Li for critically reading the manuscript.

RECEIVED FOR PUBLICATION MARCH 26, 2004; ACCEPTED JULY 5, 2004.

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Compound	structure	carbon number	structure	[0,1+,2+,3+,4+]
PBS	-	-	-	0
Tween 20	-	-	-	0
Tween 80	-	-	-	0
Pluronic F68	-	-	-	0
n-Octyl-β-D-maltoside	*n*-octyl	8	disaccharide	0
n-Octyl-β-D-maltoside	*n*-octyl	8	disaccharide	0
n-Octyl-β-D-maltoside	*n*-octyl	8	disaccharide	0
1-O-dodecyl-rac-glycerol	*n*-dodecyl	13	glycerol	0
1-O-dodecyl-rac-glycerol	*n*-dodecyl	13	glycerol	0
Phenyl-β-D-glucoside	*phenyl*	6	monosaccharide	0
Phenyl-β-D-glucoside	*phenyl*	6	monosaccharide	0
Octyl galactoside	*n*-octyl	8	monosaccharide	0
Octyl thioglucoside	*n*-octyl	8	monosaccharide	0
Octyl galactoside	*n*-octyl	8	monosaccharide	0
Octyl thioglucoside	*n*-octyl	8	monosaccharide	0
Octyl galactoside	*n*-octyl	8	monosaccharide	0
Octyl thioglucoside	*n*-octyl	8	monosaccharide	0
Dodecyl glucoside	*n*-dodecyl	12	monosaccharide	0
Dodecyl glucoside	*n*-dodecyl	12	monosaccharide	0
Dodecyl glucoside	*n*-dodecyl	12	monosaccharide	0
Dodecyl glucoside	*n*-dodecyl	12	monosaccharide	0
Sodium octyl sulfate	*n*-octyl	8	sulfate	0
Sodium octyl sulfate	*n*-octyl	8	sulfate	0
Sodium tretradecyl sulfate	*n*-tetradecyl	14	sulfate	0
Sodium tretradecyl sulfate	*n*-tetradecyl	14	sulfate	0
n-Decyl-β-D-maltoside	*n*-decyl	10	disaccharide	1
n-Decyl-β-D-maltoside	*n*-decyl	10	disaccharide	1
Sodium decyl sulfate	*n*-decyl	10	sulfate	1
Sodium decyl sulfate	*n*-decyl	10	sulfate	1
n-Tetradecyl-β-D-maltoside	*n*-tetradecyl	14	disaccharide	2
n-Tetradecyl-β-D-maltoside	*n*-tetradecyl	14	disaccharide	2
Polidocanol	*n*-dodecyl	12	polyether	2
n-Tridecyl-β-D-maltoside	*n*-tridecyl	13	disaccharide	3
n-Tridecyl-β-D-maltoside	*n*-tridecyl	13	disaccharide	3
n-Dodecyl-β-D-maltoside	*n*-dodecyl	12	disaccharide	4
n-Dodecyl-α-D-maltoside	*n*-dodecyl	12	disaccharide	4
6-Cyclohexylhexyl-β-D-maltoside	6-Cyclohexylhexy	12	disaccharide	4
Sucrose laurate	*n*-dodecyl	12	disaccharide	4
n-Dodecyl-β-D-maltoside	*n*-dodecyl	12	disaccharide	4
n-Dodecyl-β-D-maltoside	*n*-dodecyl	12	disaccharide	4
Sucrose laurate	*n*-dodecyl	12	disaccharide	4
n-Dodecyl-β-D-maltoside	*n*-dodecyl	12	disaccharide	4
Sucrose laurate	*n*-dodecyl	12	disaccharide	4
Sodium dodecyl sulfate	*n*-dodecyl	12	sulfate	4
Sodium dodecyl sulfate	*n*-dodecyl	12	sulfate	4
Sodium dodecyl sulfate	*n*-dodecyl	12	sulfate	4
Full table of compounds				
Effect of alkyl chain length for constant hydrophilic group (see plot)				
Effect of hydrophilic group types for constant alkyl chain				
Alkyl monosaccharide performance				

EXHIBIT B

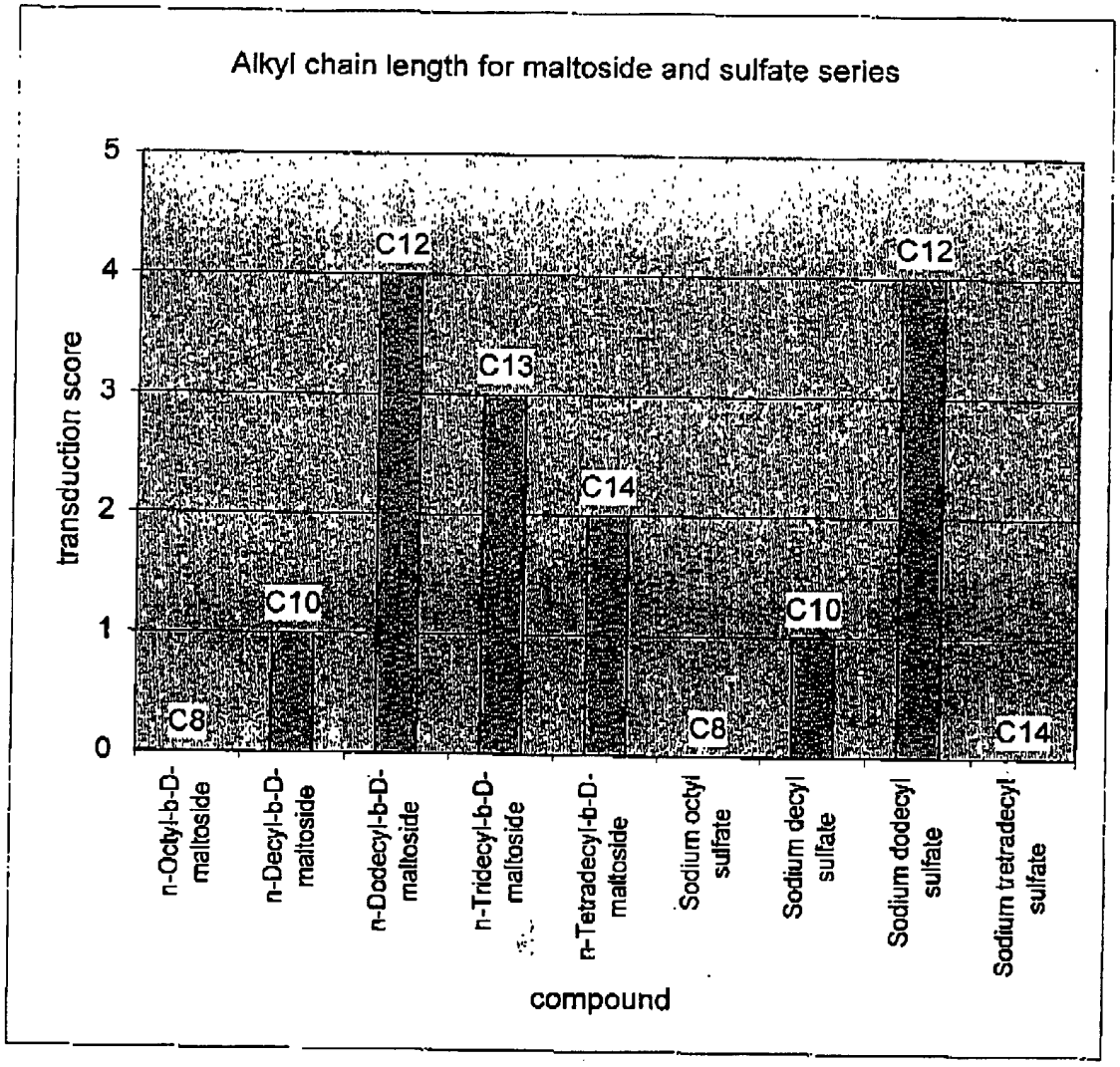
Alkyl chain length for maltoside and sulfate series
transduction score
5
4
3
2
1
0
C12
C13
C14
C10
C8
C12
C10
C8
C14
n-Octyl-b-D-maltoside
n-Decyl-b-D-maltoside
n-Dodecyl-b-D-maltoside
n-Tridecyl-b-D-maltoside
n-Tetradecyl-b-D-maltoside
Sodium octyl sulfate
Sodium decyl sulfate
Sodium dodecyl sulfate
Sodium tetradecyl sulfate
compound

EXHIBIT C

Vector	DDM		Study Ref #
	+	-	
Ad-LacZ[a]	100%	0%	P02-550-003-0019
Ad-LacZ	100%	0%	P05-014-0922
Ar20-1004[b]	122834 pg/ml[c]	6771 pg/ml[c]	P05-014-0955

[a] – Non-replicating Ad5 vector expressing β-galactosidase
[b] - Oncolytic adenoviral vector expressing murine GMCSF
[c] – Level of mGMCSF in urine after 24 hrs

EXHIBIT D

Cancer Therapy: Preclinical

CG0070, a Conditionally Replicating Granulocyte Macrophage Colony-Stimulating Factor–Armed Oncolytic Adenovirus for the Treatment of Bladder Cancer

Nagarajan Ramesh, Ying Ge, David L. Ennist, Mingzhu Zhu, Mervat Mina, Shanthi Ganesh, P. Seshidhar Reddy, and De-Chao Yu

Abstract **Purpose:** The purpose of this study was to examine the tumor specificity, cytotoxicity, and granulocyte macrophage colony-stimulating factor expression of CG0070, a conditionally replicating oncolytic adenovirus, in human bladder transitional cell carcinoma (TCC) cell lines and determine its antitumor efficacy in bladder TCC tumor models.

Experimental Design: Virus yield and cytotoxicity assays were used to determine tumor specificity and virus replication-mediated cytotoxicity of CG0070 in a panel of human bladder TCC cell lines and primary cells *in vitro*. Two s.c. and one orthotopic bladder TCC xenograft tumor models were used to assess antitumor activity of CG0070.

Results: In a matched isogenic pair of cell lines with differing retinoblastoma (Rb) pathway status, CG0070 showed selective E1a and granulocyte macrophage colony-stimulating factor (GM-CSF) expression in Rb pathway–defective cells. CG0070 replicated in Rb-defective bladder TCC cell lines as efficiently as wild-type adenovirus but produced 100-fold less virus in normal human cells. CG0070 was up to 1,000-fold more cytotoxic in Rb pathway–defective bladder TCC cells in comparison with normal human cells. Antitumor activity of CG0070 was shown in two bladder TCC s.c. xenograft tumor models following intratumoral injections and intravesical treatment in an orthotopic xenograft tumor model when compared with PBS treatment.

Conclusions: *In vitro* and *in vivo* studies showed the selective replication, cytotoxicity, GM-CSF production, and antitumor efficacy of CG0070 in several bladder TCC models, suggesting a potential utility of this oncolytic agent for the treatment of bladder cancer. Further studies are warranted to show the role of human GM-CSF in the antitumor efficacy of CG0070.

Approximately 60,000 cases of urinary bladder cancer were diagnosed and ~12,000 patients died of the disease in 2004. Roughly 75% of patients are male, making bladder cancer the fourth most common cancer affecting U.S. men (8th in women). More than 90% of bladder cancer cases are in the transitional urothelium and thus referred to as transitional cell carcinoma (TCC). Superficial bladder cancer has traditionally been treated by transurethral resection. However, patients with less differentiated large or multilocular bladder tumors and patients with carcinoma *in situ* or stage T_a and T_1 bladder cancer are at high risk for tumor recurrence and development of muscle-invasive disease or distant metastasis. In spite of early intervention with Bacillus Calmette-Guerin and transurethral resection, the high recurrence rate of bladder cancer warrants the development of new therapeutic modalities (1).

Viruses that replicate selectively in tumor cells, leading to cell lysis and release of oncolytic virions, are being developed as biological anticancer agents (2–5). The advantage of conditionally replicating selective oncolytic adenoviruses is that the virus replication in the tumor will amplify the input virus potentially leading to spread of the virus throughout the tumor mass. Additionally, tumor antigens could be released from lysed tumor cells and systemic antitumor immune response may be induced to augment the oncolytic activity. Viruses are either inherently capable of tumor-specific replication or tumor specificity can be generated through genetic manipulation of the viral genome. Of the many approaches to restrict viral replication to tumor cells, the use of human tumor-selective transcriptional regulatory elements in the control of key essential viral genes has gained immense attention (2). For adenoviruses, the early essential genes have been placed under the control of tumor-selective promoters to restrict the virus replication to target cells resulting in several tissue- or tumor-specific oncolytic agents (6–9).

Retinoblastoma (Rb) pathway defects are found in the cells of most cancers, including bladder, head and neck, colon, prostate, and melanoma (10). CG0070 is a conditionally

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Received 5/12/05; revised 8/29/05; accepted 9/6/05.
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doi:10.1158/1078-0432.CCR-05-1059

EXHIBIT E

replicating oncolytic serotype 5 adenovirus (Ad5) in which the human E2F-1 promoter, a Rb pathway–defective tumor-specific transcription regulatory element (11, 12), drives expression of the essential E1a viral genes to restrict viral replication and cytotoxicity to Rb pathway–defective tumor cells. In addition, CG0070 encodes the cDNA for human granulocyte macrophage-colony stimulating factor (GM-CSF), a cytokine known to be a potent inducer of specific, long-lasting antitumor immunity in animal models (13), under the control of the endogenous viral E3 promoter. Because the E3 promoter is activated by E1a gene product, both viral replication and GM-CSF expression may be ultimately under the control of the tumor-selective E2F-1 promoter. Due to *in situ* expression of GM-CSF, in addition to its direct oncolytic effect after local treatment, CG0070 may also induce systemic, tumor-specific immunity such that uninfected local tumor as well as distant tumor metastases may be affected.

In the current study, the tumor selectivity, cytotoxicity, and GM-CSF production and the antitumor efficacy of CG0070 have been evaluated in several bladder TCC models. In addition, the antitumor synergy of CG0070 was evaluated in combination with docetaxel in a bladder xenograft tumor model.

Materials and Methods

Cells and culture methods. Bladder TCC cell lines SW780 and RT4, human cervical carcinoma cell line HeLa-S3, human embryo lung fibroblast cell line Wi38, and the SV40 transformed, Rb-defective variant Wi38-VA13 were obtained from American Type Culture Collection (Manassas, VA). AE1-2A is an adenovirus-complementing cell line (14) and was cultured in Richter's medium containing 5% heat-inactivated fetal bovine serum. Bladder TCC cell lines 253J B-V and UC14 were kindly provided by Colin Dinney (M.D. Anderson Cancer Center, Houston, TX). The primary human cells included human aortic endothelial cells (hAEC; Cambrex BioWhittaker, Walkersville, MD) and normal human embryo lung fibroblasts (MRC-5, American Type Culture Collection). A clonal cell line, SW780-Luc, was generated by stably transducing the human bladder TCC cell line SW780 with a lentiviral vector coding for the luciferase gene product under the control of the cytomegalovirus early gene promoter/enhancer.

Virus construction and amplification. The large plasmid containing the CG0070 adenoviral genome with the designed alterations was constructed as follows: a shuttle plasmid pDr20hGmF carrying the human GM-CSF gene with the left end packaging site (ψ) was generated from recombination between plasmid pDR1F and pAr15pAE2fhGmF (15, 16). The donor plasmid pDr20hGmF was digested with *FspI*/*SpeI*. The large fragment containing the human GM-CSF cDNA was cotransformed into *E. coli* BJ5183 competent cells along with *PacI*/*SrfI*–digested pAr5pAE2fF plasmid DNA to generate full-length clone by homologous recombination (17, 18). *SwaI*-digested full-length plasmid was transfected into AE1·2a cells using the LipofectAMINE-Plus reagent system (Life Technologies, Rockville, MD). The virus was amplified in roller bottles and purified by CsCl gradient centrifugation. Ar20-1004 is identical to CG0070 except for the presence of murine GM-CSF cDNA in the E3 region instead of the human GM-CSF cDNA. Ar20-1061 is identical to CG0070 except for the absence of GM-CSF cDNA in the E3 region. For large-scale preparations, the virus was amplified in HeLa-S3 cells and purified by chromatography. Virus particle titers were determined either spectrophotometrically or by high-performance liquid chromatography as previously described (19). The infectious titers (plaque-forming units) were determined in 293 cells by plaque assay (7).

Cell viability assay and virus yield assay. Cytotoxicity assays were done on bladder TCC cell lines and nontumor cells using the Promega CellTiter aqueous nonradioactive cell proliferation kit (Promega, Inc., Madison, WI). The data were analyzed using GraphPad Prism 4 (San Diego, CA) analysis software. The virus yield assay was done as previously described (20).

E1a mRNA. E1a expression was quantitated by reverse transcription-PCR on RNA isolated using RNAzol B (Tel-TEST) as described earlier (21). The expression level of E1a for each vector was normalized to viral DNA copy number (hexon DNA copy number) determined 4 hours postinfection as described earlier (22).

GM-CSF expression. Duplicate wells of human bladder TCC cell lines were infected at the indicated particle/cell ratios for 24 hours. Cell supernatants were collected and human GM-CSF protein was quantitated by ELISA (R&D Systems, Minneapolis, MN) following the protocol of the vendor. The sensitivity of the assay was 7.8 pg/mL. Biological activity of GM-CSF was measured in TF-1 cells as previously described (15).

Efficacy studies in tumor models in nude mice. Female NCR (*nu/nu*) mice (4-6 weeks of age; body weight of 18-20 g) were purchased from Simonsen Labs (Gilroy, CA). Mice were injected s.c. in the right flank with 2×10^6 SW780 or 253J B-V cells in Matrigel (injection volume of 200 μL). When tumors reached a mean tumor volume of ~150 mm^3 [volume = $(W^2 \times L) / 2$; W, width; L, length, in cubic millimeters], animals ($n = 10$ per group) were randomly distributed into several treatment groups.

The antitumor synergy of CG0070 in combination with docetaxel was evaluated in SW780 s.c. xenograft tumor model. Monotherapy and combination group received CG0070 and docetaxel as indicated in the figure.

Tumor volume was measured twice weekly for the duration of the study beginning on day 1. Body weight was measured once per week for the duration of the study. Mice were euthanized if the tumor volume exceeded 2,000 mm^3 or if they lost >15% of their initial body weight.

Orthotopic tumors were generated on the luminal surface of the bladder by intravesically instilling human bladder TCC cell line SW780-Luc. Female NCR nude mice were anesthetized with isoflurane and a 24-gauge catheter was introduced through the urethra into the bladder. The residual urine was emptied and the bladder was flushed with PBS. One-hundred microliters of a 0.1% solution of dodecyl β-D-maltoside were then instilled into the bladder intravesically and retained for 5 minutes; after which, the bladder was washed with PBS. Dodecyl β-D-maltoside treatment was followed by an intravesical treatment with 100 μL of 0.25% solution of trypsin for 10 minutes. SW780-Luc cells (1×10^6 in 80 μL) were then administered intravesically and a purse string suture was placed around the urethral opening. The purse string was removed after 1 to 3 hours and the cells were allowed to drain.

Two weeks following cell implantation, tumor-bearing mice were anesthetized with isoflurane and a 24-gauge catheter was introduced through the urethra into the bladder. The residual urine was emptied and the bladder was flushed with PBS. Seventy microliters of a 0.1% solution of dodecyl β-D-maltoside were then instilled into the bladder intravesically and retained for 5 minutes; after which, the bladder was washed with PBS. CG0070 (3×10^{10} viral particles in 50 μL) was administered intravesically and retained in the bladder for 15 minutes. Treatment was terminated by withdrawing the virus and flushing the bladders with PBS. Body weights were monitored weekly until the end of the study. At the conclusion of the in-life phase of the study, all animals were euthanized and bladders were removed for histochemical analysis.

Histopathology and immunohistochemistry. For histologic examination, the explanted tumors or bladders were fixed in 10% buffered formalin and then 5-μm paraffin sections were either H&E stained or processed for immunohistochemical analysis and then counterstained. Antihuman cytokeratin 20 AE1 antibody staining to locate human TCC cells and hexon staining (13G9) to identify areas of virus replication were done using the DAKO ARK kit (Carpenteria, CA) following the protocol of the manufacturer. Apoptotic cells were detected using Roche *In situ* Cell Death Detection Kit (Indianapolis, IN) following the instructions of the manufacturer.

Statistical analysis. Statistical tests were done using the GraphPad Prism software (La Jolla, CA). Tukey's test was done on $\log_{10}$-transformed data to test for significance between the CG0070-treated group and the PBS-injected control in tumor model studies.

Results

Virus structure. The genomic structure of the oncolytic adenoviral vector CG0070 is shown schematically in Fig. 1A. The human E2F-1 promoter, which provides tumor specificity to any downstream gene products, was cloned in place of the endogenous E1a promoter in the Ad5 backbone. To protect from transcriptional read-through activating E1a expression, a SV40 polyadenylation signal was inserted 5′ of the E2F-1 promoter. CG0070 includes the entire wild-type E3 region except for the gp19kD-coding region. In place of the gp19kD gene, CG0070 carries the cDNA for human GM-CSF under the control of the E3 promoter. The rest of the viral vector backbone, including packaging signal, E2, E4, late protein regions, and inverted terminal repeats, is identical to the wild-type Ad5 genome. An alternate oncolytic adenovirus constructed, Ar20-1004, is identical to CG0070 except that it carries the cDNA for murine rather than human GM-CSF.

Rb status–dependent gene expression and virus replication. Measuring gene expression of CG0070 in cells with normal and defective Rb pathways provides a good measure of the Rb status dependence of the E2F-1 promoter and the selectivity of CG0070. A matched isogenic pair of cell lines, Wi38 and Wi38-VA13, which differed in their Rb pathway status, was compared for the ability to support CG0070 function as measured by expression levels of E1a and GM-CSF. Wi38-VA13 is an SV40-transformed cell derived from Wi38 in which the SV40 T antigen has disrupted the pRb-E2F pathway, resulting in high levels of free E2F transcription factor. The deregulation of the Rb pathway and up-regulation of the endogenous E2F-1 gene expression have been shown earlier in Wi38-VA13 cell line (21). The E1a mRNA level 24 hours after infection was assessed by quantitative PCR and the data were normalized to the number of Ad genome copies determined by hexon DNA levels 4 hours postinfection (Fig. 1B). Following infection with 1,000 particles per cell, an average of 2.19×10^5 copies of E1A mRNA was observed in W138 cells in comparison with 911 copies in W138-VA13 cells. A separate experiment in this cell line pair showed the dependence of GM-CSF production on a defective Rb pathway (Fig. 1B). One-thousand-fold more GM-CSF was measured in CG0070-infected Wi38-VA13 cells than in Wi38 cells. These results showed that the human E2F-1 promoter in CG0070 is capable of selectively regulating adenoviral E1a gene transcription and downstream E3 promoter–controlled human GM-CSF expression in Rb pathway–defective cells.

Following adenovirus infection of the target cells, the amount of virus produced reflects numerous processes, including the ability of a particular cell type to be infected, to transactivate promoters, to replicate the virus, and to carry out other complex activities. The production of CG0070 virus in human bladder TCC cell lines (Rb pathway defective) and human normal cells

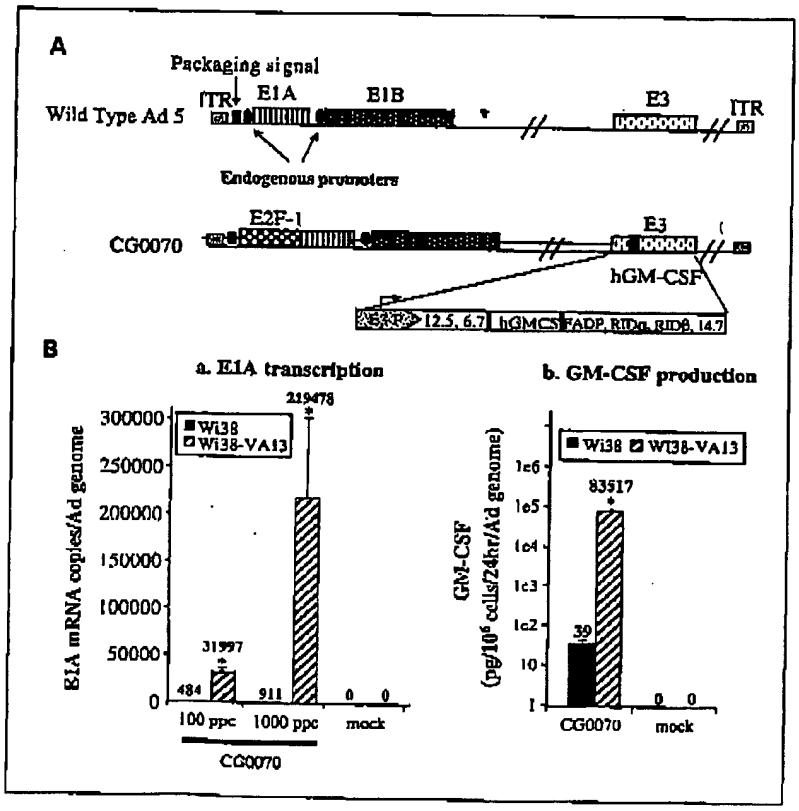

Fig. 1. Structure of CG0070 and selective expression of genes. *A*, schematic diagram of CG0070 and wild-type Ad5. CG0070 is based on Ad5 wherein the endogenous E1a promoter and E3 gp19kD-coding region have been replaced by the human E2F-1 promoter and a cDNA coding region of human GM-CSF, respectively. Complete sequencing of CG0070 was done to confirm viral genomic DNA sequence. Viral particle/plaque-forming unit ratios of the virus lots used in this study was below 30. *B*, selective E1a gene transcription and GM-CSF production in normal human embryo lung fibroblast Wi38 cells and the SV40-transformed Wi38-VA13 cells. *a*, selective E1a gene transcription. Wi38 (normal Rb pathway) and Wi38-VA13 (defective Rb pathway). *, $P < 0.01$, E1a RNA in Wi38-VA13 versus E1a RNA in Wi38 infected with the same vector (*t* test). *b*, selective GM-CSF production. Levels of human GM-CSF (*hGM-CSF*) secreted by Wi38 and Wi38-VA13 cells (100 particles per cell) were determined at 24 hours by ELISA. *, $P < 0.01$, human GM-CSF level in infected Wi38-VA13 cells versus infected Wi38 cells.

(Rb positive) was compared with that of wild-type adenovirus Ad5. CG0070 replicated in the Rb pathway–defective human bladder TCC cell lines RT4, SW780, UC14, and 253J B-V cells as efficiently as wild-type adenovirus Ad5, producing similar levels of progeny virus (3,000-9,000 plaque-forming units per cell), but was highly attenuated (~100-fold) in Rb-positive normal human cells including MRC-5 fibroblasts and hAEC primary aortic endothelial cells (Fig. 2A). CG0070 produced >2 log more progeny virus in the bladder TCC cell lines in comparison with primary cells. Under similar conditions, wild-type Ad5 produced similar levels of progeny virus in both tumor and primary cells.

Cytotoxicity in vitro. Another measure of the effectiveness of an oncolytic virus such as CG0070 is the ability to lyse tumor cells preferentially compared with normal or primary cells. Because CG0070 is designed to replicate preferentially in tumor cells that have a defective Rb pathway, tumor and normal cell lines were chosen based on this characteristic, and the cytotoxic effects of CG0070 in these cells were quantified. A panel of human Rb pathway–defective bladder TCC cell lines and normal human cells were infected with CG0070 at various multiplicities of infection (MOI). The cytotoxicity results, as assessed by the [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] assay (a quantitative cell viability assay), show that CG0070 is cytotoxic in the human bladder TCC cell lines (RT4, SW780, and UC14) but is highly attenuated in normal human cells (MRC-5 and hAEC; Fig. 2B). By day 10 following infection with 1 MOI of CG0070, for example, the viability of bladder TCC cell lines were reduced to 10% to 20% in comparison with >95% viability of normal cells. The viability of the cell lines was reduced to similar levels when infected with 100- to 1,000-fold lower levels of wild-type Ad5 (9).

GM-CSF expression in bladder TCC cells. To measure the amount of human GM-CSF expression by CG0070, bladder TCC cell lines and primary cells were infected with the virus at three different MOIs (viral particles per cell). Supernatants were collected 24 hours later and the total amount of GM-CSF protein expressed was quantified by ELISA. A general dose-response for GM-CSF expression was seen in all of the cell types tested (Table 1). The level of GM-CSF expression varied <3-fold among the CG0070-infected bladder tumor cell lines at each MOI. However, significant differences in the level of GM-CSF expression were seen between the TCC cell lines and the normal lung fibroblast cell MRC-5. For example, at an MOI of 1,000, the amount of GM-CSF protein expressed in MRC-5 cell was 17- to 45-fold lower than that expressed in TCC cell lines. The difference in GM-CSF expression was even greater at MOI of 10, where 30- to 140-fold more protein was expressed in TCC cell lines than in MRC-5. A bioassay for GM-CSF showed that the GM-CSF expressed in the bladder TCC cells was biologically active as judged by its effect on proliferating TF-1 cells (data not shown).

Table 1. Production of GM-CSF in CG0070-infected bladder TCC cells

Cell line	Viral particles/cell	ELISA (ng/10^6 cells/24 h)
RT4	1,000	1,457 ± 60
	100	381 ± 8
	10	40 ± 2
UC14	1,000	2,807 ± 145
	100	992 ± 11
	10	114 ± 3
SW780	1,000	3,853 ± 245
	100	707 ± 16
	10	214 ± 7
253J B-V	1,000	1,492 ± 101
	100	934 ± 67
	10	153 ± 2
MRC-5	1,000	85 ± 18
	100	24 ± 0.3
	10	1.5 ± 0.02

NOTE: ELISA data represent the mean ± SD of replicate wells in the units of ng/10^6 cells/24 h.

Antitumor efficacy in xenograft tumor models. The antitumor efficacy of CG0070 as a monotherapy was evaluated in two s.c. xenograft bladder TCC tumor models. S.c. tumors of bladder

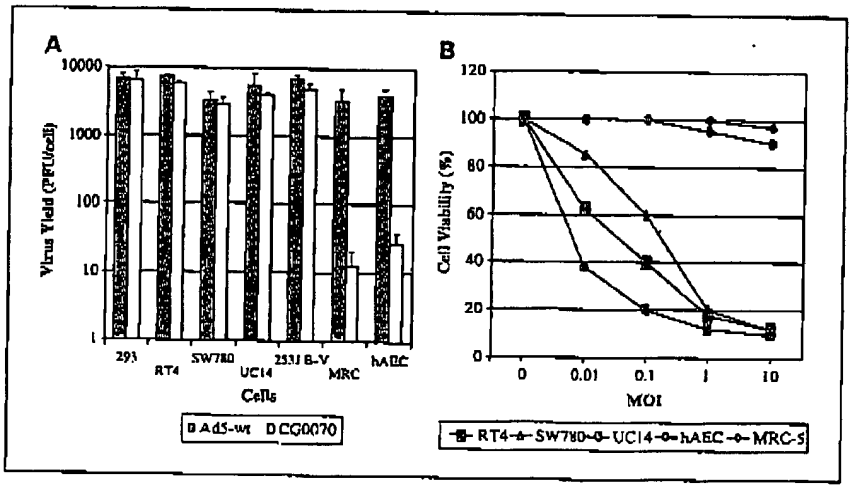

Fig. 2. CG0070 virus yield and cytotoxicity. *A*, production of CG0070 and wild-type Ad5 in human bladder TCC and normal human cells. Monolayers of 293, human bladder TCC cell lines (RT4, SW780, UC14, and 253J B-V cells), and normal human cells (MRC-5 fibroblasts and hAEC aortic endothelial cells) were infected with either CG0070 or Ad5 at an MOI of 2 plaque-forming units per cell. The cells were harvested at 72 hours after infection and viral titers were determined by plaque assay on 293 cells. The average of duplicate titers from two independent experiments was determined and normalized to titers in 293 cells. *B*, viability of cells after infection with CG0070. Human bladder TCC cells (RT4, SW780, and UC14) and human primary cells (hAEC aortic endothelial cells and MRC-5 lung fibroblasts) were infected with CG0070 at various MOIs (plaque-forming units per cell). Cell viability was determined at day 10 after infection and expressed as a percentage of the uninfected control.

TCC cell line 253J B-V were established in nude mice and were injected with three different does levels of CG0070 as indicated in the legend to Fig. 3A. Significant antitumor efficacy was observed following intratumoral injection of five doses of CG0070 at a concentration of 3×10^{10} viral particles per dose ($P < 0.0001$) as well as the two lower doses of 3×10^{9} and 3×10^{8} viral particles per dose ($P < 0.001$) compared with PBS-injected tumors. By day 60, the saline-treated tumors had increased 10.6-fold in volume whereas the CG0070-treated tumors at a concentration of 3×10^{10} viral particles per dose decreased in tumor size to 85% of the day 1 volume. At doses of 3×10^{8} and 3×10^{9} viral particles per injection, the mean tumor volume remained at the day 1 level until approximately day 43, then slowly increased to ~2-fold in volume by day 60. Significant differences in efficacy could not be observed between the three concentrations of virus that were employed in this study. No significant difference in body weight gain was evident between the control and treatment groups (data not shown). Mice treated with CG0070 at the highest dose showed complete tumor regressions in half of the treated animals (5 of 10) compared with mice treated with PBS (0 of 10).

The antitumor efficacy of CG0070 was also evaluated in s.c. SW780 bladder TCC tumor model. Significant inhibition in tumor growth was observed following five intratumoral injections of 3×10^{10} viral particles per injection of CG0070 (Fig. 3B). By day 28, on average the PBS-treated tumors increased 12-fold in volume from day 1 whereas the CG0070-treated tumors increased only 1.4-fold. The PBS-treated tumors showed an average tumor growth rate of 59.0 mm^3/d at day 28 whereas the CG0070-treated tumors showed an average tumor growth rate of only 1.9 mm^3/d at the same time point, representing a 96% inhibition of tumor growth rate ($P < 0.005$). Body weight and weight gain were generally unaffected in all the treatment groups (data not shown).

Virus infection and virus-mediated apoptosis within the tumor following intratumoral injection of CG0070 were monitored by immunohistochemical staining (Fig. 3C). The human SW780 tumor cells in the xenograft were identified following staining with antihuman cytokeratin 20 antibody. Virus replication, detected by staining for adenoviral hexon protein within the tumor mass explanted at different time points, showed significant staining for the presence of adenovirus 2 days following the final virus injection (day 12;

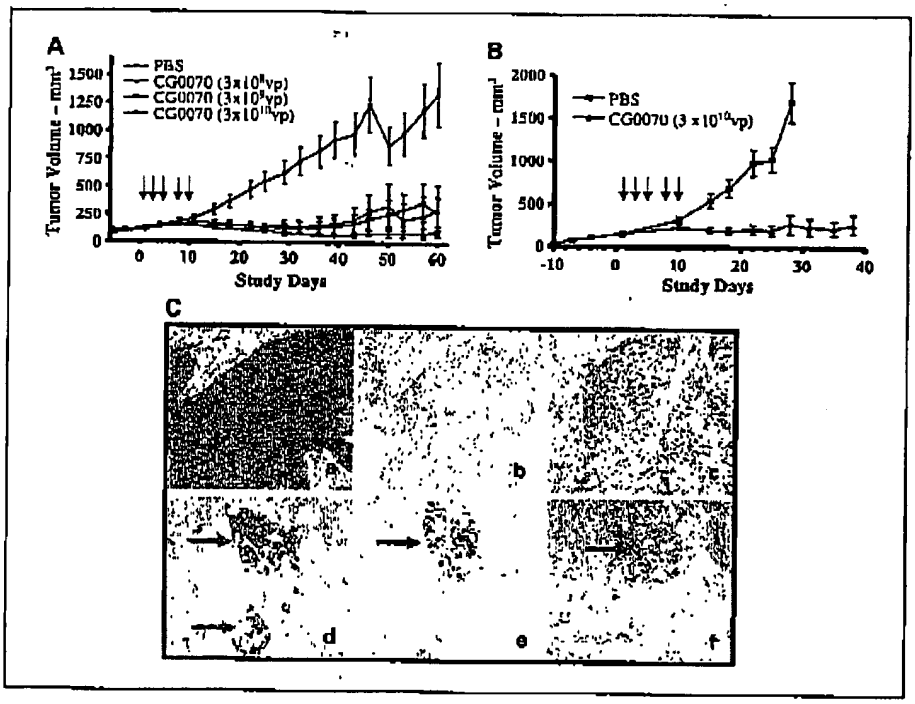

Fig. 3. Antitumor efficacy of CG0070 in bladderTCC xenograft models. *A*, 253J B-V tumor model. NCR *nu/nu* mice bearing s.c. 253J B-V tumors were injected intratumorally with PBS or CG0070 five times at a dosing volume of 50 μL, on the days indicated by the arrows (days 1, 3, 5, 8, and 10). During each dosing, CG0070 was injected intratumorally at multiple locations within the tumor. The group average tumor volumes ± SD ($n = 10$ per group) are shown for mice that received PBS, 3×10^{9}, 3×10^{9}, or 3×10^{10} viral particles per dose. All of the CG0070-treated groups were statistically different from the PBS-treated group ($P < 0.001$) on day 60. The dip in the tumor volume curve was due to the removal of four animals with large tumor in the PBS control group (one at day 43 and three at day 47) and in two other groups, one animal each with large tumor in the 3×10^{8} viral particles (day 50) and 3×10^{9} viral particles (day 57) virus treatment groups. *B*, SW780 tumor model. NCR *nu/nu* mice bearing s.c. SW780 tumors were injected intratumorally with either PBS or CG0070 on days 1, 3, 5, 8, and 10 (*arrows*). The group mean tumor volume ($n = 10$ per group) is shown for mice that received PBS or CG0070 (3×10^{10} viral particles per injection). Points, mean; bars, SE. $P < 0.005$, CG0070 versus PBS treatment. *C*, intratumoral replication of CG0070 and apoptotic cell staining. Representative tumor sections ($n = 2$). Tumors were collected on day 22 following intratumoral administration of either PBS (*a–c*) or CG0070 (*d–e*). Serial sections of the paraffin-embedded bladder tissue were used for staining. Human bladder SW780 cells were stained with antihuman cytokeratin 20 AE1 antibody (*a* and *d*). Virus replication was monitored by staining for hexon protein (*b* and *e*) in tumor sections. Apoptotic cell staining was used to locate the cells that were undergoing apoptosis within the tumor mass (*c* and *f*). Final color development in all the immunohistochemical protocols involved the use of chromogen substrate 3,3′-diaminobenzidine that results in a positive staining pattern as indicated by the arrow showing the regions of specific antibody interaction (magnification, ×20).

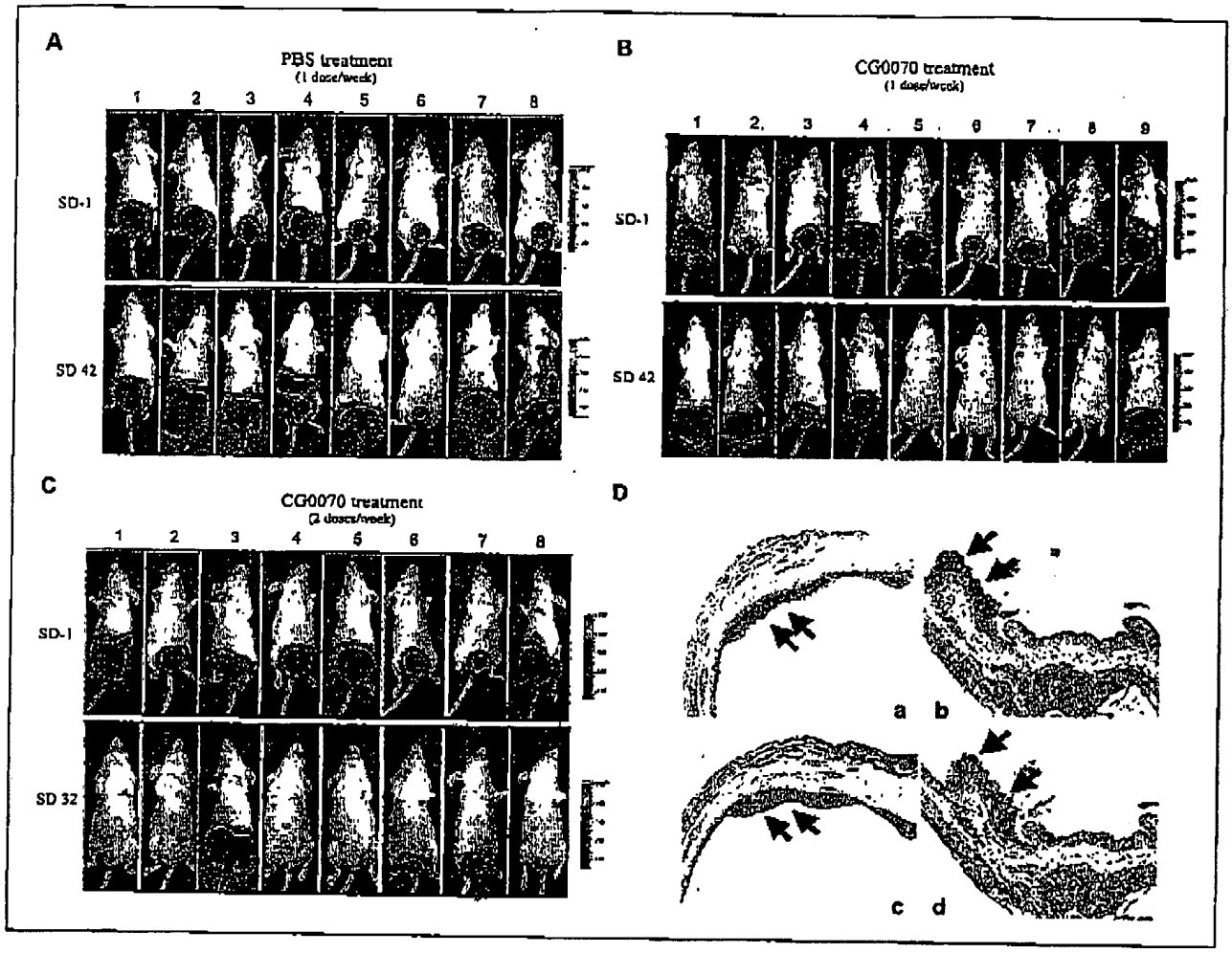

Fig. 4. Antitumor efficacy of CG0070 in SW780-Luc orthotopic bladder tumor model. *A* to *C*, following establishment of orthotopic bladder tumors, mice were treated intravesically with 50 μL of either PBS or CG0070 (3×10^{10} viral particles per dose) for a total of six doses as indicated in the figure. To monitor tumor growth, animals were imaged weekly with the Xenogen IVIS system (Alameda, CA). Images were captured with the Living Image 2.11 image software (Alameda, CA). Animals were given luciferin solution (15 mg/mL) by i.p. injection at 10 μL/g body weight (dose = 150 μg/g). The time between luciferin injection and image acquisition ranged from 5 to 15 minutes. The image shown for the animals was taken on the study days indicated in the figure. *D*, intratumoral replication of CG0070 in orthotopic SW780 bladder tumor. Representative tumor sections ($n = 4$). Bladders were harvested 24 hours following single intravesical administration of either 50 μL of PBS (*a* and *c*) or CG0070 (3×10^{10} viral particles in 50 μL; *b* and *d*). Serial sections of the paraffin-embedded bladder tissue were used for staining. Human bladder SW780 cells were stained with antihuman cytokeratin 20 AE1 antibody (*a* and *b*) and virus replication was monitored by staining for hexon protein (*c* and *d*) as indicated in Materials and Methods. Final color development in both the immunohistochemical protocols involved the use of 3,3'-diaminobenzidine that results in a positive staining pattern as indicated by the arrow showing the regions of specific antibody interaction (magnification, ×10).

data not shown). Substantial staining for hexon protein could be observed 12 days following the final virus injection (day 22) within the tumor mass. Less extensive but still demonstrable hexon staining was observed as late as day 29, which suggested that virus replication persisted for a prolonged period after virus injection (data not shown). Apoptosis associated with viral replication within the tumor mass was visualized as described in Materials and Methods. Apoptotic cell staining was observed in successive sections of tumors collected on day 22, overlapping with hexon staining, suggesting that apoptotic activity was centered in regions of viral replication.

The antitumor activity of CG0070 was further evaluated in an orthotopic bladder TCC model that more closely resembles the actual treatment setting in patients. Female NCR nude mice bearing orthotopic SW780-Luc bladder tumors received six intravesical doses of CG0070 (3×10^{10} viral particles per dose in a 50-μL dosing volume) either once weekly for 6 consecutive weeks or twice weekly for 3 consecutive weeks. Based on tumor imaging *in situ*, all of the tumors in the PBS-treated group ($n = 8$) increased in size compared with the baseline except one animal in which the tumor did not grow (Fig. 4A). In the CG0070 treatment group (one dose/wk), *in situ* tumor imaging showed that four of nine animals were tumor-free by day 42 following the initiation of treatment (Fig. 4B). The tumor in one animal increased in size, three animals were euthanized with a large tumor burden, and one animal was found dead in

the cage due, presumably to the large tumor as recorded by prior imaging data. In contrast, five of eight animals in the CG0070 treatment group (2 doses/wk) were tumor-free by day 32 following the initiation of treatment (Fig. 4C). The tumor of one animal had decreased in size (dropping from 1.45×10^6 to 5.71×10^5 photon count); that of another was stable in size; and one animal was euthanized with a large tumor burden on day 23. Immunohistochemical evaluation of the bladder sections confirmed the absence of tumor cells in CG0070-treated mice (five of eight in 2 doses/wk group and four of nine in 1 dose/wk group) deemed tumor-free by *in vivo* imaging (data not shown). Replication of virus in orthotopic tumor following intravesical treatment with CG0070 was shown by staining for hexon in histologic sections of bladder that were explanted 24 hours following virus treatment (Fig. 4D).

Several studies have shown the utility of docetaxel as a monotherapy and in combination with other chemotherapeutic agents in the treatment of metastatic bladder cancer (23, 24). Hence, the antitumor efficacy of Ar20-1004 in combination with docetaxel was assessed in the SW780 xenograft tumor model. Ar20-1004, which encodes murine GM-CSF but is otherwise identical to CG0070, was used in this study and is considered to be a murine homologue of CG0070. Animals with s.c. SW780 tumors were treated with Ar20-1004 alone, docetaxel alone, or both agents. A significant decrease in tumor volume between control and all of the treatment groups ($P < 0.0001$) was evident (Fig. 5). The PBS control group showed an average tumor growth rate of 25 ± 2.8 mm^3/d. Significant differences in efficacy were observed between the combination and monotherapy treatment groups ($P < 0.03$) with the highest antitumor efficacy obtained in the combination treatment in comparison with the monotherapy groups. The Ar20-1004 and docetaxel monotherapy groups showed an average growth rate of 7.0 ± 1.6 and 5.3 ± 0.79 mm^3/d, respectively, by day 37. In contrast, tumor volumes in mice treated with Ar20-1004 and docetaxel combination had regressed by the same time point, exhibiting an average tumor growth rate of -0.78 ± 0.35 mm^3/d. Table 2 summarizes relative tumor volume of control and treated groups on four different time points. Analysis on fractionated tumor volume indicated a synergistic effect between Ar20-1004 and docetaxel in this study (Table 2). On day 29, there was 1.35-fold improvement in antitumor activity in the combination group when compared with the expected additive effect. By day 37, the average tumor volume in Ar20-1004- or docetaxel-treated mice increased further compared with the tumor volumes in mice treated with both agents. The tumor growth inhibition in the combination treatment group was 3.3-fold higher over an additive effect following treatment with either agent alone.

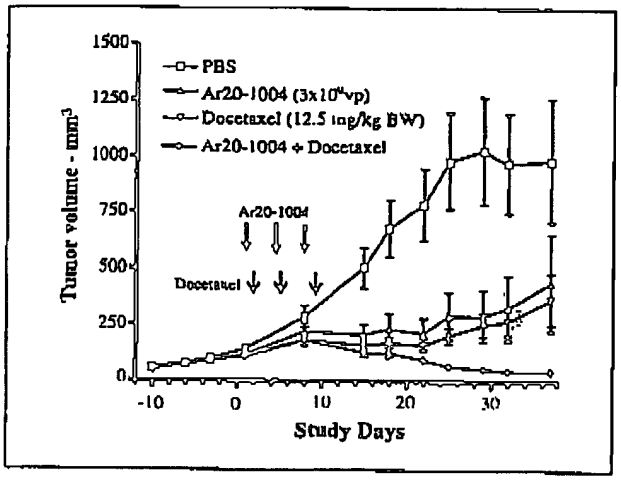

Fig. 5. Synergistic antitumor efficacy of Ar20-1004 in combination with docetaxel in the s.c. SW780 bladder TCC xenograft model. NCR *nu/nu* mice bearing s.c. SW780 tumors were injected intratumorally with PBS or Ar20-1004, at a dosing volume of 50 μL, on days 1, 4, and 8 or i.v. docetaxel (100 μL) on days 2, 5, and 9 (*arrows*) as indicated in the figure. The group mean tumor volume ($n = 10$ per group) is shown for mice that received PBS, Ar20-1004 (3×10^8 viral particles/injection), docetaxel (12.5 mg/kg/body weight/injection), or both Ar20-1004 and docetaxel. Points, mean; bars, SE. $P < 0.001$, Ar20-1004 and docetaxel combination versus Ar20-1004 or docetaxel alone treatment. The dip in the tumor volume curve was due to the removal of four animals with large tumor in the PBS control group (one at day 25, two at day 28, and one at day 32).

Discussion

Rb pathway defects are found in a majority of tumors, including TCC of bladder. Altered pRb (absent pRb or mutated pRb) expression has been associated with bladder cancer progression and patients with bladder tumors that have altered pRb protein products are at a high risk of recurrence and death (25). In some patients, overexpression of pRb has been observed in bladder tumors and has been suggested to be indicative of dysfunctional Rb status through upstream changes in the cell cycle pathway involving Rb phosphorylation leading to functional inactivation of pRb (26). Deletion of p16, a key protein in the Rb pathway, has been reported in 30% to 70% of TCC and is found in all tumor stages and grades. Furthermore, abnormalities of p16 are reported to be a poor prognostic marker associated with a high rate of recurrence in patients with superficial TCC of the bladder (27). Because tumor cells do not usually develop both Rb and p16 mutations, the additive prevalence of Rb and p16 gene mutations results in nearly uniform inactivation of the Rb pathway in TCC of the bladder (28).

CG0070 is a conditionally replicating oncolytic Ad5 adenovirus designed to preferentially replicate in and kill cancer cells. Tumor selectivity of CG0070 is based on the use of the E2F-1 promoter to control viral replication. The E2F-1 promoter mediated transgene expression has been previously shown to be selective in Rb pathway–defective tumor cells (15, 16). In this communication, we have examined the Rb status dependence of gene expression of CG0070 and its potential application in the treatment of bladder TCC. *In vitro* studies showed that following infection with CG0070, 100 times more E1a mRNA and 1,000 times more GM-CSF were detected in the Rb pathway–defective cells compared with normal cells. This suggested that the expression of E1a gene in CG0070, which is under the control of the E2F-1 promoter, as well as the transgene in the E3 region is highly dependent on the Rb status of the cells. Further, viral replication and cytotoxicity of CG0070 were significantly enhanced in Rb pathway–defective cells. In Rb pathway–defective bladder TCC cells, CG0070 produces >100-fold more viruses in tumor cells in comparison with the normal cells. CG0070 is highly cytotoxic to the bladder tumor cells with minimal cytolysis of normal cells. Even when the tumor cells are infected with CG0070 at an MOI

Table 2. Combination treatment with Ar20-1004 and docetaxel

SD*	Ar20-1004	Docetaxel	FTV relative to untreated controls† combination treatment		Ratio of expected FTV/observed FTV‡
			Expected§	Observed	
15	0.409	0.317	0.129	0.232	0.556
22	0.269	0.205	0.055	0.105	0.524
29	0.282	0.243	0.069	0.051	1.353
37	0.446	0.371	0.165	0.050	3.300

*Study day after first treatment with the virus.
†Fractional tumor volume (FTV) calculated as mean tumor volume experimental/mean tumor volume control.
‡Mean FTV of Ar20-1004 × mean FTV of docetaxel
§Obtained by dividing the expected FTV by the observed FTV. A ratio of >1 indicates a synergistic effect and a ratio of <1 indicates a less than additive effect.

of 1, almost all the cells are lysed by day 10 following infection, compared with minimal cell killing observed with CG0070 in primary cells such as MRC5 and hAEC even at an MOI of 10. *In vivo* studies with CG0070 showed the strong antitumor activity of the virus in bladder TCC xenograft tumor models and showed significant antitumor synergy when combined with the chemotherapeutic agent docetaxel. Previous studies with E2F-1 promoter–controlled oncolytic adenoviruses have shown similar tumor cell specificity and cytotoxicity *in vitro* in Rb pathway–defective cells in comparison with normal cells (15, 16, 21, 29, 30). In addition, these viruses showed significant antitumor efficacy in several s.c. xenograft tumor models in mice. Several genomic structural features distinguish CG0070 from these E2F-1 controlled viruses including the absence of an endogenous E1a promoter, inclusion of a SV40 Poly(A) sequence 5′ to the packaging signal to avoid any nonspecific transcriptional initiation, which may increase specificity, unchanged location of the packaging signal with respect to the wild-type Ad5 virus to avoid potential recombinants, and inclusion of the GM-CSF transgene in the E3 gp19kD position under the control of the E3 promoter.

CG0070 is also intended to selectively produce GM-CSF in Rb pathway–defective tumor cells due to the dependence of the E3 promoter that drives GM-CSF expression on transactivation by E1A. CG0070-mediated GM-CSF expression was observed in all bladder TCC cell lines examined. At 100 viral particles per cell or higher, production of biologically active GM-CSF exceeded 40 ng/mL/10^6 cells/24 h (Table 1), a level that Dranoff et al. (13) report as sufficient to induce potent, long-lasting antitumor immunity in *in vivo* tumor vaccination models. Therefore, CG0070 has the potential to produce GM-CSF in quantities predicted to be therapeutic. The antitumor effects observed in the present study are likely entirely due to the oncolytic activity of CG0070 because the human GM-CSF encoded by this virus is not biologically active in mice (31). Optimal demonstration of enhancement of antitumor responses by treatment with CG0070 would necessitate testing the vectors in immunocompetent animal models with Ar20-1004, which carries the mouse GM-CSF transgene. Nevertheless, in the absence of a robust immunocompetent murine animal model, studies in immune-deficient nude mice have shown that an oncolytic adenovirus similar to CG0070, but expressing murine GM-CSF instead of human GM-CSF, enhances antitumor activity in comparison with the parental non-GM-CSF-expressing vector, presumably through the activation of innate immune responses that are maintained in nude mice (15, 16). Histochemical examination of the virus-injected tumors showed that both vectors induced necrosis and mononuclear cell infiltration into the tumor but only the murine GM-CSF-expressing adenovirus resulted in eosinophil infiltration. Similar mononuclear cell infiltrations were observed following intravesical treatment of bladder of immunocompetent mice only with Ar20-1004 but with an identical virus to Ar20-1004 that does not encode the GM-CSF transgene (data not shown). It is anticipated that CG0070 will have greater efficacy in immunocompetent humans wherein the GM-CSF expressed by the virus has the potential to activate an immunologically specific antitumor immune response.

Intravesical administration of a therapeutic agent would greatly assist in maximizing the drug delivery to the target tumor tissue with minimum distribution to the vital organs outside of the bladder. Hence, bladder cancer has been treated traditionally through the intravesical instillation of therapeutic agents such as Bacillus Calmette-Guerin, chemotherapeutic agents, and gene therapeutic agents. Local delivery of an armed oncolytic adenovirus in the bladder would allow for efficient infection of the superficial tumors by adenovirus without significant systemic exposure and thus restrict the transgene expression from the infected tumor cells wherein the virus replicates with minimal overall circulating levels of the transgene. In addition to the antitumor efficacy observed in s.c. xenograft tumor models with CG0070 either as a single agent or in combination with docetaxel, results from the studies done in the orthotopic tumor model in nude mice showed the antitumor potential of CG0070 in a model treatment setting. Two phase I trials have been done with nonreplicating adenovirus-based vectors that show the relative safety and feasibility of intravesical instillation of virus (32, 33). One of the limiting factors in these phase I trials for the treatment of bladder cancer has been the low infectivity of the bladder epithelium with adenovirus vectors due to the barrier imposed by the polyanionic glycosaminoglycan layer (34, 35). Pretreatment of the bladder with the transduction enhancement agents such as Syn3 and dodecyl β-D-maltoside may enhance the infectivity of adenovirus in tumors with consequently improved clinical efficacy (36, 37).

Immunotherapy with Bacillus Calmette-Guerin, delivered intravesically into the bladder, results in a massive local immune response characterized by the induction of proinflammatory cytokines in the urine and in bladder tissue (38).

Studies in immunocompetent murine models have shown the role of different lymphocyte subpopulations in the antitumor activity of Bacillus Calmette-Guerin. Under normal conditions, very few leukocytes can be detected in the suburothelial stroma of the bladder following Bacillus Calmette-Guerin instillation; significant influx of various leukocyte subpopulations and chemokines is thought to probably contribute to the antitumor effect. An oncolytic virus expressing GM-CSF may be particularly effective in eliciting such a response because the cell death resulting from virus replication will release tumor antigens that will then induce a GM-CSF-mediated immune response.

In summary, we have shown that the E2F-1 promoter in CG0070 tightly controls the expression of viral E1a gene and GM-CSF, resulting in high tumor selectivity of CG0070 towards Rb pathway–defective bladder TCC cells. Production of biologically active GM-CSF is induced in a dose-related fashion at levels known to stimulate antitumor immunity in the tumor vaccine setting. Significant antitumor efficacy has been shown in two s.c. xenograft bladder TCC tumor models and an orthotopic TCC tumor model in mice as a monotherapy and in combination with docetaxel in one xenograft tumor model. These *in vitro* and *in vivo* findings show the strong antitumor activity of armed oncolytic adenovirus CG0070 and support its evaluation for the treatment of bladder cancer in humans.

Acknowledgments

We thank Gail Colbern and Melinda VanRooy and her group for help in the conduct of animal studies, Marina Abramova for histology and immunohistochemistry, Jun Liu and Debbie Farson for the generation of the SW780-Luc cells, Mebratu Gebreyesus and Joseph Wypych for the production of viruses, and Yuanhao Li for critical review of the manuscript.

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